REMARKS

Claims 1 and 4-23 were presented for examination, and were rejected. Claims 20 and 23 have been amended. The amendments focus the method claims on certain conditions disclosed in the specification, at paragraphs [0004], [0005], [0045], and [0046]. The amendments add no new matter.

These amendments remove a basis for the rejection of claim 20, by removing a term that the Examiner recited in an enablement rejection. They remove the only stated basis for rejection of claim 23, which is now drawn to a use that the Examiner acknowledged as being enabled. Therefore, they are believed to place the claims in condition for allowance or at least in better condition for appeal. Entry of the amendment is therefore respectfully requested.

The first sentence of the specification is also amended, to correct the year in the filing date of the corresponding provisional application. The correct filing date is readily ascertained from the application number of the provisional application, and is reflected on the application data sheet filed with the application. Therefore, correction of the year in that date adds no new matter.

Reconsideration in view of the amendments and the following remarks is respectfully requested.

Rejections based on 35 U.S.C. § 112.

Claims 20 and 23 were rejected under 35 U.S.C. § 112, first paragraph because the scope of the claims was allegedly broader than the scope of enablement. Those claims have been amended to recite specific conditions associated with TGF-β that were disclosed in the specification. The use of TGF-β inhibitors for treating these conditions was known in the art prior to the filing date of this application, as evidenced by the following references, attached hereto:

Exhibit A: U.S. Patent 5,824,655, issued on October 20, 1998, provides gene therapy approaches to reduce the activity of TGF- β and describes their use to treat "various fibrotic diseases and conditions." (Abstract) It also says, "Methods to control the action of TGF- β will

have a significant impact on the treatment and prevention of tissue fibrosis and on the overall cost of healthcare." (Col. 1, lines 56-59).

Exhibit B: WO 02/29105, published on April 11, 2002, describes methods to inhibit TGF- β and applications of this to "a method for treating fibrosis." It further states in paragraph [0004], "In view of the wide-ranging activities of TGF-β, it is clear that overactivity of TGF- β is implicated in the conditions of fibrosis, defects in cell proliferation, and coagulation defects. Thus, a factor which inhibits the activity of TGF- β would be extremely useful in treating conditions associated with the overactivity of TGF- β."

Exhibit C: Akhurst, R.J., *J. Clin. Invest.* 109:1533-36 (2002), identified as Volume 109, No. 12, June 2002, is entitled "TGF- β antagonists: Why suppress a tumor suppressor?" It says, "A large number of papers have provided strong evidence for a role of TGF- β in tumor invasion or metastasis (1-6). Now, two papers in this issue of the *JCI* highlight this clinically significant action of TGF- β in tumorigenesis and provide very encouraging results regarding both the efficacy and the low toxicity of a soluble TGF- β receptor that effectively reduces tumor spread (7,8)." (Col. 1, first paragraph.)

Exhibit D: Slawomir, W-P., *Invest. New Drugs* 21:21-32 (2003), identified as issue 1, for February 2003, says this: "Neutralization of TGF- β or inhibition of its production is an effective method of cancer treatment in variety of animal models. Several agents targeting TGF- β are in the early stages of development and include anti-TGF- β antibodies, small molecule inhibitors of TGF- β, Smad inhibitors and antisense gene therapy." (Abstract) It further describes "TGF- β Inhibitors in development." (Table 2, page 24). It also concludes that "Preclinical studies demonstrated significant antitumor activity of agents targeting this molecule as well as synergy with immunostimulating agents and traditional chemotherapeutics. It is difficult to assess objectively which of the multiple effects of TGF- β contributes the most to tumor growth and metastasis. Clearly, agents that block TGF- β production, secretion, activation and metabolism should be extensively investigated as a new therapeutic modality for all types of solid tumors." (Last paragraph of the article, page 28.)

The Examiner alleged that the applicant was claiming treatment of all manner of inflammation, cancer, and other conditions. However, the claims recite treatment of disorders associated with excessive TGF-β activity, and are thus limited to use for that category of conditions: they do not attempt to encompass all types of inflammation, for example, regardless of origin or mediation. Furthermore, the Exhibits teach that inhibitors of TGF- β activity are clinically effective in a variety of cancer models, and that such compounds show great promise "for all types of solid tumors". They state that such inhibitors are effective to treat various fibrotic conditions and cell proliferation defects, and they demonstrate that TGF- β inhibitors are active in multiple animal models and in preclinical studies against a broad array of tumors. In view of these teachings, one of ordinary skill would have been able to practice the invention for at least the treatment of the TGF-β associated aspects of fibroproliferative disorders and cancer, as recited in amended claim 20. In view of the amendment and the knowledge available in the art before the application was filed, the claims are believed to be commensurate in scope with the disclosure, and to be enabled at least for use as claimed; therefore, withdrawal of this rejection is respectfully requested.

RejectionsBased on 35 U.S.C. § 102

Claims 1-18, and 20-22 were rejected under 35 U.S.C. § 102(b) over Cai et al., WO 02/47690. The Examiner asserts that Cai teaches:

> several pyrimidine compounds, which include compounds, compositions and the method of use claimed in the instant claims. See formula I on page 10 and note when A=C, given the same pyrimidine core, all variable groups overlap with those of the instant claims. Especially see formula II and III on page 11-12 which shows the desired pyrimidine compounds. See entire document for the details of the invention. See pages 13-34 for various species of pyrimidine compounds. See pages 139-152 for a Table of compounds. See especially compounds 73, 75, 46 and 20.

The applicant respectfully traverses this rejection. First, the Examiner is applying an anticipation standard that is contrary to well-established case law and the standards in the MPEP. Formula I in Cai represents a genus that discloses an enormous, and perhaps infinite, number of compounds. A reference does not anticipate a claim even if the genus it discloses overlaps that claim, unless the reference discloses a specific compound or discloses the claimed genus with

specificity. (*See* MPEP 2131.02. *See also* MPEP 2131.03, stating that overlap of ranges is not enough to show anticipation.) For example, if a broad genus anticipated all species it could conceivably encompass, the patenting of a species or subgenus within the scope of the broad genus would not be permitted; yet such selection inventions are routinely allowed. *See* MPEP 2144.08.II, describing patentability analysis for a species or subgenus encompassed by a broad genus. The genus represented by Formula I in Cai is extremely broad: for example, Ar_2 can be any "optionally substituted aryl or heteroaryl", and R1 and R2 are also disclosed with similar breadth. Formula I, which includes a seemingly infinite number of compounds, was not shown to disclose any species or subgenus with sufficient specificity to anticipate the present claims.

Formula II in Cai is only slightly narrower than formula I; for example, Ar_2 can still be any "optionally substituted aryl or heteroaryl", and again R1 and R2 are disclosed with the same scope as above. Accordingly, formula II, which includes a vast number of compounds, does not disclose any species or subgenus with sufficient specificity to anticipate the present claims.

Formula III is irrelevant to the present claims: it requires the aryl group on the amino group at position 4 of the pyrimidine ring to be a phenyl group, while the claims are drawn to compounds having selected heterocycles in that position, and do not allow the corresponding group to be phenyl. Therefore, the claims are entirely distinct from Formula III in Cai.

Similarly, the specific compounds identified by the Examiner (compounds 73, 75, 46 and 20 in the Table) all have a phenyl ring linked to the pyrimidine by a Nitrogen at position 4 of the pyrimidine ring, and thus those compounds cannot anticipate the present claims.

The Examiner also points to the last five compounds on page 22 and the first five compounds on page 34, and the last species on page 33. However, each of those species includes a 3-pyridyl group as the substituent at position 2 of the pyrimidine, while the claims require a phenyl group at that position. Therefore, none of the species identified by the Examiner falls within the scope of the claims. Accordingly, the anticipation rejections based on Cai must be withdrawn.

The applicant appreciates withdrawal of the anticipation rejections based on Davies, et al. and the anticipation rejections based on Kleeman, et al.

Rejections based on 35 U.S.C. § 103

Claims 1-23 were rejected as obvious over Cai, et al., 'for reasons of record'. The applicant traverses this rejection, also for reasons of record, which are repeated and further elaborated below.

The applicant traverses this rejection. As demonstrated above, Cai does not disclose any species within the scope of the claims. Its disclosure of a broad class of pyrimidine compounds is insufficient to anticipate or render obvious the claimed genus, and none of the species identified by the Examiner falls within the scope of the claims.

Furthermore, Cai teaches features of a preferred subgenus that guide the reader away from the genus of the present claims; for example, it teaches that the C-2 group on the pyrimidine is preferably a heterocyclic group (see Formula III, where the preferred forms of Ar1 include one or two nitrogen atoms), and it teaches that the aryl group on the N at position 4 of the pyrimidine is preferably phenyl (see, e.g, formula III). Each of these directs the person of ordinary skill away from the genus of the present claims. Therefore, there is no motivation from the general teaching in Cai to make compounds within the present claims.

The Examiner says that Cai teaches the "equivalency of those compounds exemplified with specific substituents with that generically recited on page 10-13 for Formula I-III," and alleges that it "would have been obvious to one having ordinary skill in the art at the time of the invention was made to make pyrimidine compounds variously substituted with Ar1, Ar2, R1, R2 and R3 as permitted by the reference and expect resulting compounds (instant compounds) to possess the uses taught by the art in view of the equivalency teaching outlined above."

The Examiner has provided no rationale for or explanation for the assertion that Cai teaches "equivalency" of its compounds with each other or with other compounds in the genus that

Cai generically embraces. Cai does not, in fact, teach 'equivalency' either expressly or implicitly. As stated in the previous response, the applicant understands this comment from the Examiner to mean that the Examiner considers the disclosure of a genus to be an assertion that all species within it are equivalent, or that disclosure of features in a Markush group used to define a genus indicates that those features are 'equivalent'. As shown before, and as further explained below, that is an improper standard and cannot be used to assert that such groups or compounds are 'equivalent'.

As stated in the previous Office Action response, this rejection applies a clearly improper legal standard by effectively asserting that a broad genus renders obvious all species that it could read on. That is directly contradicted by the MPEP: please see e.g., MPEP 2144.08, especially Section II, which specifically relates to obviousness analysis in chemical inventions. It states: "The fact that a claimed species or subgenus is encompassed by a prior art genus is not sufficient by itself to establish a *prima facie* case of obviousness. *In re Baird*, 29 USPQ2d 1550 (Fed. Cir. 1994)." Furthermore, *In re Ruff*, 256 F.2d 590, 118 USPQ 340 (CCPA 1958), which is cited in MPEP 2144.06, says, "The mere fact that components are claimed as members of a Markush group cannot be relied upon to establish the equivalency of these components." The Examiner has not provided any basis to consider the compounds disclosed in Cai, or their structural elements, to be 'equivalent'; it certainly does not disclose such equivalence, since it teaches the opposite; and the MPEP and case law expressly state that the inclusion in a Markush group of various groups is insufficient to show that they are either 'equivalent' *or* obvious. Accordingly, the Examiner has not established a *prima facie* case of obviousness.

Furthermore, the reference viewed as a whole, as it must be, teaches that the structural features in the Markush groups in Cai are NOT considered equivalent *even by the authors of the reference*: the evidence in the reference proves the non-equivalence of the compounds and structural features in Cai. And its teachings guide one of ordinary skill away from the compounds and classes of the present claims for at least the reasons described above, which is evidence that the claimed invention is not obvious in view of Cai.

MPEP 2144.08 requires the Examiner, when analyzing the obviousness of a claim, to "Consider the size of the Genus" in the prior art, and to "Determine whether one of ordinary skill in the art would have been motivated to select the claimed species or subgenus." It requires the Examiner to "consider the number of variables that must be selected or modified, and the nature and significance of the differences between the prior art and the claimed invention." The Examiner has not given those factors due consideration, as demonstrated by the lack of structural analysis in the rejections. If the mere inclusion of multiple alternatives in a Markush established their "equivalency", there would be no reason to consider either the size of the prior art genus or any motivation to 'select' the claimed species or the number of variables that must be selected from a prior art genus to arrive at the claimed invention: a disclosed genus would anticipate and render obvious all it encompasses. That, however, is not the law of obviousness for chemical inventions, as the MPEP makes abundantly clear. *In re Ruff,* which is cited above and quoted in MPEP 2144.06, expressly rejects that basis for asserting equivalency.

The Examiner indicates that Cai renders obvious all "pyrimidine compounds variously substituted with Ar1, Ar2, R1, R2 and R3 as permitted by the reference", indicating that all such compounds are considered equivalent and thus obvious. The MPEP expressly requires consideration of the number of features that would have to be selected to arrive at the claimed invention, and of the size of the prior art genus, and of the guidance the reference provides toward or away from the claimed genus. The Examiner therefore must consider the teachings of preferred features in the reference, for example:

the preferred Ar_1 in Cai is a nitrogen-containing heterocyclic group (see para. [0026], where preferred species of Formula III have at least one of B, D, E, F, and G equal to Nitrogen);

the preferred Ar_2 in Cai is a phenyl ring (see para. [0025]: Preferred compounds of formula I are...Formula III", which has a phenyl ring for Ar_2).

Neither of those is consistent with or encompassed by the claimed genus, and each of them guides the reader away from major structural features of the claimed compounds. Considering

the size of the broad genus disclosed in Cai, and the number of variables that would have to be selected to approach the genus of the present claims, and that at least these major ones would have to be selected *contrary to the express teachings in Cai*, Cai does not render obvious the claimed genus under the standards that must be applied. Accordingly, this rejection should be withdrawn.

Claims 1-19 and 22 were again rejected as obvious based on Kleeman, et al., U.S. Patent No. 5,849,758. The Examiner stated that Kleeman "teaches several pyrimidine compounds which include instant compounds." The Examiner further stated, "Kleeman et al. teaches the equivalency of those compounds exemplified with specific substituents with that generically recited for formula I....Although applicants' amendment to exclude O from X definition obviates the 102 rejection, in view of equivalency teaching of X as O with X as S in Kleeman, this rejection is proper and is maintained."

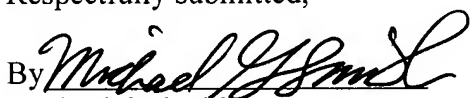
The applicant traverses this rejection. As has been explained above, and in the previous response to this rejection, the mere inclusion in a Markush of multiple alternatives does not establish that those alternatives are "equivalent" (*In re Ruff*). The Examiner cited nothing from Kleeman that would suggest O and S were considered "equivalent" when used in its compounds. Indeed, Kleeman exemplifies only compounds wherein X represents O, as far as the applicant's representative can determine; for example, see the generic structure for each of the Tables in Kleeman, they all represent compounds wherein X represents Oxygen. In addition, Kleeman specifically states that "Particularly good results in control of weeds are achieved with compounds wherein X represents an oxygen atom." Thus Kleeman provides express guidance *away* from X = S: it does NOT teach or suggest that X = Sulfur is "equivalent" to X = Oxygen, it expressly teaches *non*-equivalence of those alternatives for X. In view of the express teaching in Kleeman that X=S is <u>not</u> equivalent to X=O, combined with the scope of the genus in Kleeman, and the number of additional variables that would have to be selected to arrive at the claimed invention, Kleeman does not render the claimed genus obvious under the proper legal standards that must be applied. Accordingly, this rejection should be withdrawn.

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to withdraw the outstanding rejection of the claims and to pass this application to issue. If it is determined that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number given below.

In the event the U.S. Patent and Trademark office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to Deposit Account No. 03-1952 referencing Docket No. **219002029400**. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Dated: August 11, 2006

Respectfully submitted,

By 

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WO 02/29105 A1

(54) Title: INHIBITION OF TGF-β AND USES THEREOF

(57) Abstract: The present invention provides a method for inhibiting activity of TGF-β, comprising contacting tissue expressing TGF-β with an amount of ebaf or an ebaf analogue. The present invention further provides a method for treating a condition associated with overactivity of TGF-β, particularly fibrosis, a defect in cell proliferation, or a coagulation defect. The present invention also provides a method for inhibiting activity of TGF-β, comprising contacting tissue expressing TGF-β with a modulator of ebaf expression, or a modulator of expression of an ebaf analogue. The present invention is further directed to a method for treating fibrosis in a subject in need of treatment, comprising administering to the subject an amount of ebaf or an ebaf analogue effective to treat the fibrosis. Finally, the present invention provides a method for treating a defect in cell proliferation in a subject in need of treatment, comprising administering to the subject an amount of ebar or an ebaf analogue effective to treat the defect in cell proliferation.

INHIBITION OF TGF-β AND USES THEREOF

Statement of Government Interest

[0001] This invention was made with government support under NIH Grant No. CA8466. As such, the United States government has certain rights in this invention.

Background of the Invention

[0002] Transforming growth factor-beta (TGF-β) is a pleiotropic peptide that controls proliferation and differentiation of many cell types, and modulates the coagulation process. Many cells synthesize TGF-β, and almost all of them have specific receptors for this peptide.

[0003] The activities of TGF-β are well documented. TGF-β inhibits the growth of epithelial cells (Böttinger *et al.*, 1997; Hall *et al.*, 1996; Kornmann, 1999; Morton and Barrack, 1995; and Mur *et al.*, 1998), but promotes the proliferation of fibroblasts (Bettinger *et al.*, 1996; Clark *et al.*, 1997; and Franzen and Dahlquist, 1994) and the deposition of collagen (Bettinger *et al.*, 1996; and Han, 1999). In particular, TGF-β has been implicated in various forms of fibrosis, including: normal wound healing and scar formation (Choi *et al.*, 1996; Lin *et al.*, 1995; Liu *et al.*, 1995; Messadi, 1998; O'Kane and Ferguson, 1997; and Stelnicki *et al.*, 1998); the formation of keloid (Lee, 1999; Tredget *et al.*, 1998; Younai *et al.*, 1994; and Zhang *et al.*, 1995); radiation-induced fibrosis (Randall and Coggle, 1996); fibromatosis (Berndt *et al.*, 1995; and Zamora *et al.*, 1994); hypertrophic burn scars (Polo *et al.*, 1997; and Zhang *et al.*, 1995); pulmonary fibrosis (Khalil *et al.*, 1996; Martinet *et al.*, 1996; Specks *et al.*, 1995; Vaillant *et al.*, 1996; and Yoshida and Hayashi, 1996), including that associated with radiation (Yi *et al.*, 1996), drugs (Coker *et al.*, 1997; and Zhang *et al.*, 1996), and transplantation (El-Gamel *et al.*, 1998); the healing of the myocardial infarct (Hao *et al.*, 1999); fibrosis associated with autoimmune disorders such as scleroderma (Querfeld *et al.*, 1999); and sarcoidosis (Salez *et al.*, 1998).

[0004] In view of the wide-ranging activities of TGF-β, it is clear that overactivity of TGF-β is implicated in the conditions of fibrosis, defects in cell proliferation, and coagulation defects. Thus, a factor which inhibits the activity of TGF-β would be extremely useful in treating those conditions associated with the overactivity of TGF-β. However, there are currently no known effective inhibitors of TGF-β.

[0005] Recently, a new member of the TGF-β superfamily, lefty-1, was recognized for its distinct asymmetric expression in gastrulating mouse embryos (Meno *et al.*, 1996; and Oulad-Abdelghani *et al.*, 1998). Lefty-A is the human homologue of lefty-1. Lefty-A is also known as endometrial bleeding associated factor (ebaf) protein, which is associated with abnormal endometrial bleeding (Kothapalli *et al.* (1997).

[0006] The *ebaf* gene is located on human chromosome 1, at band q42.1, and its nucleotide and deduced amino acid sequences are known. *Ebaf* is highly expressed in human endometrium prior to and during menstrual bleeding or abnormal uterine bleeding (Kothapalli *et al.*, 1997). The *ebaf* gene is also expressed in certain adenocarcinomas that exhibit mucinous differentiation, including colonic, duodenal, ovarian, and testicular carcinomas (Tabibzadeh *et al.*, 1997). The amino acid sequence of the ebaf protein shows homology with, and structural features of, members of the TGF-β superfamily (Kothapalli *et al.*, 1997), and ebaf is also recognized as a member of the TGF-β superfamily.

[0007] In view of the similarity in the nucleotide sequences of *lefty-1* and *ebaf,* Kosaki *et al.* (1999) hypothesized, and subsequently showed, that mutations in the *ebaf* gene are associated with left-right axis malformations in humans. During the course of this investigation, a second human gene, *lefty-B,* was identified. In mice, both the *lefty-1* gene and the *lefty-2* gene reside on chromosome 1H2. In humans, both the *lefty-A* (*ebaf*) gene and the *lefty-B* gene map to human syntenic region 1q42, and are separated from each other by 50 kb. The nucleotide sequences of *lefty-A* (*ebaf*) and *lefty-B* are 97% identical, so

these proteins are more closely related to each other than to either of the mouse homologues.

[0008] Human ebaf proteins are derived from a precursor with an approximate molecular weight of 42 kD. Polypeptides with approximate molecular weights of 34 kD and 28 kD are secreted by cells, along with the precursor. RGKR and RHGR are the cleavage sites, respectively, for the 34-kD and 28-kD protein forms. Lefty proteins are secreted in glycosylated form.

Summary of the Invention

[0009] The present invention is based upon the discovery that ebaf inhibits activity of TGF-β. On the basis of this finding, the present invention provides a method for inhibiting activity of TGF-β, comprising contacting tissue expressing TGF-β with an amount of ebaf or an ebaf analogue effective to inhibit the activity of TGF-β.

[0010] The present invention further provides a method for treating a condition associated with overactivity of TGF-β in a subject in need of treatment, comprising contacting tissue expressing TGF-β in the subject with an amount of ebaf or an ebaf analogue effective to inhibit activity of TGF-β, thereby treating the condition.

[0011] The present invention also discloses a method for inhibiting activity of TGF-β, comprising contacting tissue expressing TGF-β with a modulator of ebaf expression, or a modulator of expression of an ebaf analogue, in an amount effective to induce or enhance expression of ebaf or the ebaf analogue, thereby inhibiting the activity of TGF-β.

[0012] The present invention is further directed to a method for treating fibrosis in a subject in need of treatment, comprising administering to the subject an amount of ebaf or an ebaf analogue effective to treat the fibrosis.

[0013] Finally, the present invention provides a method for treating a defect in cell proliferation in a subject in need of treatment, comprising

administering to the subject an amount of ebaf or an ebaf analogue effective to treat the defect in cell proliferation.

[0014] Additional objects of the present invention will be apparent in view of the description which follows.

Brief Description of the Figures

[0015] Figure 1 provides a model for TGF-β signaling. TGF-β functions through binding to two receptors, and signals through the Smad family of transcription factors. The biological effects of the members of the TGF-β family are signaled through two classes of molecules, designated as type I and type II receptors. These are transmembrane serine-threonine kinases that share homology with each other, but have distinctive features. The dimerized ligand first binds the type II receptor; the type I receptor is subsequently recruited, leading to the formation of a heteromeric complex. Within this complex, the type II receptor, which is constitutively active, phosphorylates the type I receptor in the GS (glycine-serine rich) domain (Padgett *et al.*, 1998).

[0016] Figure 2 depicts the nucleotide sequence and corresponding amino acid sequence for ebaf.

[0017] Figures 3A-3E demonstrate inhibition of transcriptional activity of TGF-β by ebaf. P19 cells were transfected with reporter genes (A, B: pSBE-luciferase; C: p21-luciferase; D: Cdc25-luciferase; E: CTGF-luciferase). Twenty-four hours after transfection, cells were treated with TGF-β and/or ebaf for 30 min. Cells were removed 24 h after treatment, and analyzed for luciferase activity. Values presented are the means of triplicate determinations ± standard deviations.

[0018] Figures 4A and 4B depict inhibition of TGF-β-mediated nuclear translocation of Smad2 and Smad4 by ebaf. P19 cells were treated for 30 min, with medium alone (control), TGF-β (5 ng/ml), recombinant ebaf (5 ng/ml), or TGF-β (5 ng/ml) plus ebaf (5 ng/ml). A: The cytosolic and nuclear fractions were prepared from the treated cells, and equal amounts of protein (10 g/lane)

were subjected to Western blot analysis for Smad2, Smad4, Smad5, and Histone 3. The localization of Histone 3 in the nuclear fraction, and the absence of Histone 3 from the cytosolic preparation, show that these preparations were not cross-contaminated. Arrows point to the accumulated Smad2 and Smad4 in the nuclear lysates. B: Smad4 was localized by immunoperoxidase staining in the treated cells. Arrows point to nuclear Smad4. panel a: control cells treated with medium alone; panel b: cells treated with TGF-β; panel c: cells treated with ebaf; panel d: cells treated with TGF-β and ebaf; percentage of cells showing nuclear staining: control: 2%; TGF-β: 25%; ebaf: 3%; and TGF-β + ebaf: 1%

[0019] Figure 5 depicts inhibition of TGF-β-mediated heterodimerization of Smad2 and Smad4 by ebaf. P19 cells were treated for 30 min with medium alone (control), TGF-β (5 ng/ml), ebaf (5 ng/ml), or TGF-β (5 ng/ml) plus ebaf (5 ng/ml). The proteins in the nuclear preparations were immunoprecipitated with an antibody to Smad2, and the immunoprecipitates were subjected to Western blot analysis for Smad4 (upper panel). The cell lysates were analyzed for Smad2 and Smad4 by Western blotting, in order to assess the overall amount of each protein (two lower panels). IP: immunoprecipitation; WB: Western blotting

[0020] Figure 6 illustrates inhibition of TGF-β-mediated phosphorylation of Smad2 by ebaf. P19 cells were treated for 30 min in culture medium alone (control), or in culture medium supplemented with TGF-β (5 ng/ml), recombinant ebaf (5 ng/ml), or TGF-β (5 ng/ml) plus ebaf (5 ng/ml), in the presence of 0.15 μCi [^{32}P]-orthophosphate per ml. Smad2 was immunoprecipitated with Smad2 antibody, and the immunoprecipitate was subjected to SDS-PAGE followed by autoradiography (upper panel). The overall amount of Smad2 was assessed by Western blotting of cell lysates (lower panel). RI: TGF-β receptor type I; WB: Western blotting; ^{32}P: [^{32}P]-orthophosphate; Rx: radioactivity.

[0021] Figures 7A and 7B demonstrate inhibition by ebaf of transcriptional activity of constitutively-active TGF-β receptor type I. A: P19 cells were transfected with pSBE-Lux reporter construct. Twenty-four hours after transfection, cells were treated for 30 min with cell culture medium alone (control), or medium supplemented with TGF-β (5 ng/ml), recombinant ebaf (5 ng/ml), or TGF-β (5 ng/ml) plus ebaf (5 ng/ml). Cells were removed 24 h after treatment, then analyzed for luciferase activity. Values presented are the means of triplicate determinations ± standard deviations. B: P19 cells were transfected with the constitutively-active TGF-β receptor type I. Transfected cells then were treated for 30 min, in the presence of 0.15 μCi [^{32}P]-orthophosphate (10 μCi/ml), with cell culture medium alone (control), or medium supplemented with TGF-β (5 ng/ml), ebaf (5 ng/ml), or TGF-β (5 ng/ml) plus ebaf (5 ng/ml). Smad2 was immunoprecipitated, and the immunoprecipitates were subjected to SDS-PAGE followed by Western blotting and autoradiography (upper panel). The overall amounts of TGF-β receptor type I and Smad2 were assessed by Western blotting (two lower panels). RI: TGF-β receptor type I; WB: Western blotting; ^{32}P: [^{32}P]-orthophosphate; IP: immunoprecipitation

[0022] Figures 8A and 8B demonstrate competition between ebaf and TGF-β for binding to TGF-β receptor type I. P19 cells were incubated with radioiodinated TGF-β (10 μCi/ml) in the presence of varying concentrations of ebaf. The receptor-bound TGF-β then was cross-linked to the receptor in the presence of DSS. A: The cell lysates were subjected to immunoprecipitation, using an antibody to TGF-β receptor type I, and then SDS-PAGE, followed by Western blotting and autoradiography (upper panel). The overall amount of cell radioactivity was assessed by subjecting a total amount of 1 μCi/lane from the cell lysate to SDS-PAGE and autoradiography (middle panel). The overall amount of TGF-β receptor was analyzed by Western blotting (lower panel). B: The Smad2 proteins in the cell lysates were immunoprecipitated, and the immunoprecipitates were subjected to SDS-PAGE and autoradiography (upper panel). The overall amount of radioactivity was assessed by subjecting the cell

lysate (1 μCi/lane) to gel electrophoresis and autoradiography (second panel from top). The total amounts of TGF-β receptor type I and Smad2 were analyzed by Western blotting (two lower panels). RI: TGF-β receptor type I; WB: Western blotting; ^{32}P: [^{32}P]-orthophosphate; IP: immunoprecipitation; Rx: radioactivity

[0023] Figures 9A and 9B illustrate the effect of ebaf on Smad7 and total protein synthesis. A: P19 cells were treated for 30 min with cell culture medium supplemented with varying concentrations of ebaf, as shown. The proteins in the cell lysates were subjected to Western blot analysis for Smad7. Equal loading was assessed by Western blotting the cell lysates for actin. B: P19 cells were transfected with a constitutively-active form of TGF-β receptor type I. Twenty-four hours after transfection, cells were treated with cyclohexamide (20 g/ml) for 1 h. Cells then were treated for 30 min with culture medium alone (control), or medium supplemented with TGF-β (5 ng/ml), ebaf (5 ng/ml), or TGF-β (5 ng/ml) plus ebaf (5 ng/ml). Smad2 was immunoprecipitated from the cell lysates, and the immunoprecipitates were subjected to SDS-PAGE and autoradiography (upper panel). The overall amount of radioactivity was assessed by gel electrophoresis and autoradiography of the cell lysates (1 μCi/lane) (lower panel). WB: Western blotting; ^{32}P: [^{32}P]-orthophosphate; IP: immunoprecipitation

[0024] Figure 10 depicts activation of the MAPK pathway by ebaf-conditioned media. upper panel: 293 cells were transfected with the mutated GGKG (amino acid residues 74-77) and GHGR (amino acid residues 132-135) forms of ebaf. The conditioned media were used for the treatment of P19 cells, and activation of the MAPK pathway was visualized *in vivo* using the PathDetect™ reporting system. middle panel: Culture media of 293 cells transfected with empty pcDNA3 vector or ebaf were incubated with the indicated concentrations of anti-ebaf antibody, A351, to block the activity of ebaf in the medium. The media were used to treat the pluripotent mouse P19 embryonal carcinoma cells. Activation of the MAPK pathway was analyzed *in*

vivo using the luciferase reporting system, as indicated in Materials and Methods. lower panel: P19 cells were incubated with the indicated concentrations of recombinant ebaf corresponding to the 28-kD form of ebaf (amino acid residues S137-P366). Activation of the MAPK pathway was visualized *in vivo* using the PathDetect™ reporting system, as indicated in Materials and Methods. The error bars show the standard deviation for three different experiments.

[0025] Figure 11 demonstrates activation of the MAPK pathway by recombinant ebaf. P19 cells were incubated with 15 ng/ml of the recombinant ebaf for the indicated durations. As a positive control, cells were treated with 15 ng/ml of epidermal growth factor (EGF) for 15 min. After cytokine treatment, activated phosphorylated (upper panel) and total (lower panel) forms of p42/p44-kD MAPK were visualized by Western blotting, as indicated below.

[0026] Figure 12 depicts the appearance of CCD19Lu cells treated with the medium from wild-type 293 cells (-), and those treated with the medium of 293 cells producing ebaf (+). The CCD19Lu cells had a normal appearance, and exhibited a spindle cell morphology. In contrast, most CCD19Lu cells treated with the culture medium were lost. Few cells that remained attached to the dish had abnormal morphology. Some cells were round, and had detached from the dish (arrows).

[0027] Figure 13 depicts the results of the MTT assay of CCD19Lu cells treated with 293 conditioned medium (-ebaf) or 293 ebaf-containing medium (+ebaf) 9 days after initial treatment. The MTT assay was performed as described in the text, and results are expressed as relative optical densities.

[0028] Figure 14 illustrates the effect of ebaf on the growth of fibroblastic cells *in vivo*. GE+E86 cells were transduced with a retroviral vector expressing green fluorescent protein (LG) or green fluorescent protein (GFP) and ebaf (LEIG). Cells were grown in culture. After 24 h, conditioned media were tested for the presence of ebaf. Ebaf was secreted by LEIG cells, but not by LG cells. 5

x 10^6 LG cells (panels A-D) or LEIG cells (panels E-F) were injected subcutaneously into nu/nu mice at the upper left (UL), upper right (UR), lower left (LL), and lower right (LR) aspects of the back. Each animal also was injected intraperitoneally with 5 x 10^6 cells. Three weeks after injection, the animals were sacrificed. The subcutaneous tumors (marked by black lines in panels A, C, and E) were removed; these are shown in panels B, D, and F. The thoracic and abdominal organs were removed en bloc; these are shown to the right of the subcutaneous tumors in panels B, D, and F. The peritoneal tumors are marked by white lines. The volume of each subcutaneous tumor then was determined. volumes of subcutaneous tumors: panel B: UL: 3.5 cc, UR: 2 cc, LL: 2.5 cc, LR: 4 cc; panel D: UL: 2 cc, UR: 3.9 cc, LL: 1.2 cc, LR: 1.3 cc; panel F: UL: 0.6 cc, UR: 0.7 cc, LL: 0.7 cc, LR: 0.5 cc

Detailed Description of the Invention

[0029] The present invention provides a method for inhibiting TGF-β activity, comprising contacting tissue expressing TGF-β with an amount of ebaf effective to inhibit the activity of TGF-β. Unless otherwise indicated, "ebaf" includes both an ebaf (lefty-A) protein and an "ebaf analogue". As used herein, ebaf protein has the amino acid sequence set forth in Figure 2. An "ebaf analogue" is a functional variant of the ebaf protein, having ebaf-protein biological activity, that has 80% or greater (preferably, 90% or greater) amino-acid-sequence homology with the ebaf protein, as well as a fragment of the ebaf protein having ebaf-protein biological activity. As used herein, the term "ebaf-protein biological activity" refers to protein activity which inhibits activity of TGF-β, as disclosed below. Additionally, the term "ebaf analogue", as defined herein, includes peptides related to ebaf that exert similar ebaf-protein biological activity, particularly lefty-B, lefty-1, and lefty-2 proteins, and preferably lefty-B. Ebaf may be produced synthetically or recombinantly, or may be isolated from native cells; however, it is preferably produced

recombinantly, using cDNA encoding ebaf (Figure 2), along with conventional techniques.

[0030] The method of the present invention may be used to inhibit activity of TGF-β *in vitro* or *in vivo*. As used herein, the term "inhibit activity of TGF-β" means inhibit the signaling mechanisms of TGF-β, as disclosed herein, including Smad2 phosphorylation, Smad2/4 heterodimerization, and Smad2 and Smad4 nuclear translocation, as well as associated downstream signaling. Inhibition of these signaling mechanisms by TGF-β may be detected by known procedures, including any of the methods, molecular procedures, and assays disclosed herein.

[0031] In accordance with the methods of the present invention, ebaf or an ebaf analogue may be contacted with tissue expressing TGF-β by introducing to the tissue the ebaf or ebaf analogue protein itself, or by introducing to the tissue a nucleic acid encoding ebaf or the ebaf analogue in a manner permitting expression of ebaf or ebaf analogue protein. Expression of TGF-β may be detected in tissue by detection methods readily determined from the known art, including, without limitation, immunological techniques (*e.g.*, binding studies and Western blotting), hybridization analysis (*e.g.*, using nucleic acid probes), fluorescence imaging techniques, and/or radiation detection.

[0032] Ebaf protein or ebaf analogue protein may be introduced to tissue expressing TGF-β *in vivo* in a subject by known techniques used for the introduction of proteins, including, for example, injection and transfusion. The subject is preferably a mammal (*e.g.*, humans, domestic animals, and commercial animals), and is most preferably a human. When tissue expressing TGF-β is localized to a particular portion of the body of the subject, it may be desirable to introduce the protein directly to the tissue by injection or by some other means (*e.g.*, by introducing ebaf or an ebaf analogue into the blood or another body fluid). The amount of ebaf protein or ebaf analogue protein to be used is an amount effective to inhibit activity of TGF-β, and may be readily determined by the skilled artisan.

[0033] In the method of the present invention, ebaf or an ebaf analogue also may be introduced to tissue expressing TGF-β by introducing into a sufficient number of cells of the tissue a nucleic acid encoding ebaf or the ebaf analogue, in a manner permitting expression of ebaf or the ebaf analogue. The nucleic acid may be introduced using conventional procedures known in the art, including, without limitation, electroporation, DEAE Dextran transfection, calcium phosphate transfection, monocationic liposome fusion, polycationic liposome fusion, protoplast fusion, creation of an *in vivo* electrical field, DNA-coated microprojectile bombardment, injection with recombinant replication-defective viruses, homologous recombination, gene therapy, viral vectors, and naked DNA transfer, or any combination thereof. Recombinant viral vectors suitable for gene therapy include, but are not limited to, vectors derived from the genomes of viruses such as retrovirus, HSV, adenovirus, adeno-associated virus, Semiliki Forest virus, cytomegalovirus, and vaccinia virus. The amount of ebaf protein or ebaf analogue protein to be used is an amount effective to inhibit activity of TGF-β. This amount may be readily determined by the skilled artisan.

[0034] It is also within the confines of the present invention that a nucleic acid encoding ebaf or an ebaf analogue may be introduced into suitable cells *in vitro* using conventional procedures. Cells expressing ebaf or the ebaf analogue then may be introduced into a subject to inhibiting activity of TGF-β *in vivo*. To reduce rejection, the cells are preferably removed from the subject, subjected to DNA techniques to incorporate nucleic acid encoding ebaf or the ebaf analogue, and then reintroduced into the subject.

[0035] The ability of ebaf to inhibit activity of TGF-β renders ebaf particularly useful for treating conditions associated with overactivity of TGF-β. As used herein, "overactivity of TGF-β" includes pathologic activity of TGF-β and pathologic expression of TGF-β in a particular tissue, as compared with normal activity of TGF-β and normal expression of TGF-β in the same type of tissue. It is believed that, by inhibiting activity of TGF-β, ebaf and ebaf

analogues will be useful for the treatment of conditions associated with the overactivity of TGF-β. It is further believed that ebaf and ebaf analogues would be effective either alone or in combination with therapeutic agents, such as chemotherapeutic agents or antiviral agents, which are typically used in the treatment of these conditions.

[0036] Accordingly, the present invention provides a method for treating a condition associated with overactivity of TGF-β in a subject in need of treatment, comprising contacting tissue expressing TGF-β in the subject with an amount of ebaf or an ebaf analogue effective to inhibit activity of TGF-β, thereby treating the condition. As described above, the subject is preferably a mammal (*e.g.*, humans, domestic animals, and commercial animals), and is most preferably a human.

[0037] In the treatment of a condition associated with overactivity of TGF-β, ebaf or an ebaf analogue may be contacted with tissue expressing TGF-β by introducing to the tissue the ebaf protein or ebaf analogue protein itself, in accordance with known methods, including injection, transfusion, and any methods described above. For example, when tissue expressing TGF-β is localized to a particular portion of the body of the subject, it may be desirable to introduce the protein directly to the tissue by injection or by some other means (*e.g.*, by introducing ebaf or an ebaf analogue into the blood or another body fluid). The amount of ebaf protein or ebaf analogue protein to be used is an amount effective to inhibit activity of TGF-β, as defined above, and may be readily determined by the skilled artisan.

[0038] Alternatively, in accordance with known methods, including those described above, ebaf or an ebaf analogue may be contacted with tissue expressing TGF-β by introducing to the tissue a nucleic acid encoding ebaf or an ebaf analogue, in a manner permitting expression of ebaf protein or ebaf analogue protein. The nucleic acid may be introduced using conventional procedures known in the art, including, without limitation, electroporation, DEAE Dextran transfection, calcium phosphate transfection, monocationic

liposome fusion, polycationic liposome fusion, protoplast fusion, creation of an *in vivo* electrical field, DNA-coated microprojectile bombardment, injection with recombinant replication-defective viruses, homologous recombination, gene therapy, viral vectors, and naked DNA transfer, or any combination thereof. Recombinant viral vectors suitable for gene therapy include, but are not limited to, vectors derived from the genomes of viruses such as retrovirus, HSV, adenovirus, adeno-associated virus, Semiliki Forest virus, cytomegalovirus, and vaccinia virus. The amount of ebaf protein or ebaf analogue protein to be used is an amount effective to inhibit activity of TGF-β, as defined above. This amount may be readily determined by the skilled artisan.

[0039] Overactivity of TGF-β may be associated with such conditions as fibrosis, defects in cell proliferation, and coagulation defects. In the method of the present invention, ebaf or an ebaf analogue may be used to treat forms of fibrosis, including, without limitation, the following: scars, particularly scars caused by burning, radiation, chemicals, or myocardial infarct; keloid; cirrhosis; Asherman's syndrome; Meigs' syndrome; muscular dystrophies, particularly Duchenne muscular dystrophy; autoimmune disorders leading to fibrosis, particularly scleroderma; post-surgical fibrosis, particularly fibrosis induced by surgery or surgical manipulation; fibrosis induced by non-surgical manipulation; and primary pulmonary fibrosis, particularly Hamman Rich Syndrome and retroperitoneal fibrosis. In accordance with the method of the present invention, ebaf or an ebaf analogue may further be used to treat defects in cell proliferation, including, without limitation, hyperplasia and neoplasia. Finally, ebaf or an ebaf analogue also may be used to treat coagulation defects, including menstrual bleeding, abnormal uterine bleeding, coagulopathies, and toxemia of pregnancy.

[0040] The present invention further provides a method for inhibiting activity of TGF-β in tissue, comprising contacting tissue expressing TGF-β with a modulator of ebaf expression, or a modulator of expression of an ebaf analogue, in an amount effective to inhibit the activity of TGF-β. Examples of such

modulators of expression include, but are not limited to, retinoic acid, estrogen, or progesterone.

[0041] The present invention also provides a method for treating a subject having fibrosis, comprising administering to the subject an amount of ebaf or an ebaf analogue effective to treat fibrosis. The subject is preferably a mammal (*e.g.*, humans, domestic animals, and commercial animals), and is most preferably a human. As described above, fibrosis includes, without limitation, scars, particularly scars caused by burning, radiation, chemicals, or myocardial infarct; keloid; cirrhosis; Asherman's syndrome; Meigs' syndrome; muscular dystrophies, particularly Duchenne muscular dystrophy; autoimmune disorders leading to fibrosis, including scleroderma; post-surgical fibrosis, including fibrosis induced by surgery or surgical manipulation; fibrosis induced by non-surgical manipulation; and primary pulmonary fibrosis, including Hamman Rich Syndrome and retroperitoneal fibrosis.

[0042] The ebaf or ebaf analogue of the present invention is administered to a subject in need of treatment for fibrosis in an amount which is effective to treat the fibrosis. As used herein, the phrase "effective to treat the fibrosis" means effective to ameliorate or minimize the clinical impairment or symptoms of the fibrosis. For example, where the fibrosis is Duchenne muscular dystrophy, the amount of ebaf or ebaf analogue effective to treat the fibrosis is that which can ameliorate or minimize the symptoms of Duchenne muscular dystrophy, including proximal muscle weakness and lack of co-ordination. The amount of ebaf or ebaf analogue effective to treat fibrosis in a subject in need of treatment will vary depending upon the particular factors of each case, including the type of fibrosis, the stage of fibrosis, the subject's weight, the severity of the subject's condition, and the method of administration. This amount can be readily determined by the skilled artisan.

[0043] According to the method of the present invention, ebaf or an ebaf analogue may be administered to a human or animal subject by known procedures, including, without limitation, oral administration, parenteral

administration, transdermal administration, and administration through an osmotic mini-pump. Preferably, the ebaf or ebaf analogue is administered orally. The ebaf or ebaf analogue of the present invention also may be administered to a subject in accordance with any of the above-described methods for effecting *in vivo* contact between tissue and ebaf or an ebaf analogue.

[0044] For oral administration, the formulation of ebaf or ebaf analogue may be presented as capsules, tablets, powders, granules, or as a suspension. The formulation may have conventional additives, such as lactose, mannitol, corn starch, or potato starch. The formulation also may be presented with binders, such as crystalline cellulose, cellulose derivatives, acacia, corn starch, or gelatins. Additionally, the formulation may be presented with disintegrators, such as corn starch, potato starch, or sodium carboxymethylcellulose. The formulation also may be presented with dibasic calcium phosphate anhydrous or sodium starch glycolate. Finally, the formulation may be presented with lubricants, such as talc or magnesium stearate.

[0045] For parenteral administration, ebaf or an ebaf analogue may be combined with a sterile aqueous solution which is preferably isotonic with the blood of the patient. Such a formulation may be prepared by dissolving a solid active ingredient in water containing physiologically-compatible substances, such as sodium chloride, glycine, and the like, and having a buffered pH compatible with physiological conditions, so as to produce an aqueous solution, then rendering said solution sterile. The formulations may be presented in unit or multi-dose containers, such as sealed ampoules or vials. The formulation may be delivered by any mode of injection, including, without limitation, epifascial, intracapsular, intracutaneous, intramuscular, intraorbital, intraperitoneal, intraspinal, intrasternal, intravascular, intravenous, parenchymatous, or subcutaneous.

[0046] For transdermal administration, ebaf or an ebaf analogue may be combined with skin penetration enhancers, such as propylene glycol,

polyethylene glycol, isopropanol, ethanol, oleic acid, N-methylpyrrolidone, and the like, which increase the permeability of the skin to the ebaf or ebaf analogue, and permit the ebaf or ebaf analogue to penetrate through the skin and into the bloodstream. The ebaf/enhancer or ebaf analogue/enhance compositions also may be further combined with a polymeric substance, such as ethylcellulose, hydroxypropyl cellulose, ethylene/vinylacetate, polyvinyl pyrrolidone, and the like, to provide the composition in gel form, which may be dissolved in solvent such as methylene chloride, evaporated to the desired viscosity, and then applied to backing material to provide a patch.

[0047] The ebaf or ebaf analogue of the present invention also may be released or delivered from an osmotic mini-pump. The release rate from an elementary osmotic mini-pump may be modulated with a microporous, fast-response gel disposed in the release orifice. An osmotic mini-pump would be useful for controlling release, or targeting delivery, of ebaf or ebaf analogue.

[0048] The present invention also provides a method for treating a defect in cell proliferation in a subject in need of treatment, comprising administering to the subject an amount of ebaf or an ebaf analogue effective to treat the defect in cell proliferation. Examples of defects in cell proliferation include, without limitation, hyperplasia and neoplasia. As used herein, "hyperplasia" refers to the abnormal multiplication or increase in the number of normal cells, in normal arrangement, in a tissue. Moreover, as used herein, "neoplasia" refers to the uncontrolled and progressive multiplication of cells under conditions that would not elicit, or would cause cessation of, multiplication of normal cells. Neoplasia results in the formation of a "neoplasm", which is defined herein to mean any new and abnormal growth, particularly a new growth of tissue, in which the growth is uncontrolled and progressive. Neoplasms include benign tumors and malignant tumors (*e.g.*, carcinomas, lymphocytic leukemias, myeloid leukemias, lymphomas, melanomas, sarcomas, etc.). Malignant neoplasms are distinguished from benign in that the former show a greater degree of anaplasia, or loss of differentiation and orientation of cells, and have the properties of

invasion and metastasis. Thus, neoplasia includes "cancer", which herein refers to a proliferation of cells having the unique trait of loss of normal controls, resulting in unregulated growth, lack of differentiation, local tissue invasion, and metastasis.

[0049] In the method of the present invention, ebaf or an ebaf analogue is administered to a subject in need of treatment for a defect in cell proliferation in an amount which is effective to treat the defect in cell proliferation. As used herein, the phrase "effective to treat the defect in cell proliferation" means effective to ameliorate or minimize the clinical impairment or symptoms of the defect in cell proliferation. For example, where the defect in cell proliferation is neoplasia, the clinical impairment or symptoms of the neoplasia may be ameliorated or minimized by diminishing any pain or discomfort suffered by the subject; by extending the survival of the subject beyond that which would otherwise be expected in the absence of such treatment; by inhibiting or preventing the development or spread of the neoplasm; or by limiting, suspending, terminating, or otherwise controlling the maturation and proliferation of cells in the neoplasm. The amount of ebaf or ebaf analogue effective to treat a defect in cell proliferation in a subject in need of treatment will vary depending on the particular factors of each case, including the type of defect in cell proliferation, the stage of the defect in cell proliferation, the subject's weight, the severity of the subject's condition, and the method of administration. This amount can be readily determined by the skilled artisan.

[0050] According to the method of the present invention, ebaf or an ebaf analogue may be administered to a human or animal subject by any known procedures, including all of those described above. Preferably, the ebaf or ebaf analogue is administered orally. The ebaf or ebaf analogue of the present invention also may be administered to a subject according to any of the above-described methods for effecting *in vivo* contact between tissue and ebaf or an ebaf analogue.

[0051] The present invention is described in the following Experimental Details section, which is set forth to aid in the understanding of the invention, and should not be construed to limit in any way the scope of the invention as defined in the claims which follow thereafter.

Experimental Details

1. Ebaf Inhibits TGF-β

(a) *Introduction*

[0052] Numerous lines of evidence support the view that the biological signaling of TGF-β, and members of its superfamily, is mediated through a class of cytoplasmic proteins which are designated as Smads (for *C. elegans sma* and *Drosophila "Mothers against decapentaplegic" Mad* genes) (Derynck *et al.*, 1998). TGF-β functions through binding to two receptors, and signals through the Smad family of transcription factors (Figure 1).

[0053] Three families of Smads have been identified (Kretzschmar and Massagué, 1998). The first family is comprised of the receptor-bound Smads, or R-Smads. These proteins are directly phosphorylated by the receptor kinases.

[0054] The second family includes proteins which are not direct receptor substrates, but which associate with the R-Smads; these are called Co-Smads. The third family of proteins – the Anti-Smads – inhibits the activation of the R-Smads. In vertebrates, the R-Smads include Smads 1, 2, 3, 5, and 8, the Co-Smads include Smad 4, and the Anti-Smads include Smads 6 and 7 . (Kretzschmar and Massagué, 1998).

[0055] The specificity of the signaling by members of the TGF-β superfamily is mediated by the type of R-Smads which they activate. In the case of TGF-β and activin, this includes Smads 2 and 3; for the bone morphogenic proteins (BMPs), it includes Smads 1, 5, and 8. Smad 4 acts as the common Smad that, by virtue of binding to the R-Smads, forms a heteromeric complex that is translocated to the nucleus. Within the nucleus, this complex binds a DNA-binding protein which, in the case of TGF-β and activin, is called Fast-1

(Kretzschmar and Massagué, 1998). Within this complex, Smad 4 is essential for the transcriptional activity of the promoter responsive to the ligand (*e.g.*, PAI-1 up-regulation in response to TGF-β).

[0056] The ability of TGF-β to inhibit the activity of such kinase complexes derives in part from its regulatory effects on the cyclin-dependent kinase inhibitors, p21/WAF1/Cip1, p27Kip1, and p15. Upon treatment of cells with TGF-β, these three inhibitors bind to, and block the activities of, specific cyclin-cyclin-dependent kinase complexes, causing cell-cycle arrest. Little is known, however, of the mechanism through which TGF-β activates these cyclin-dependent kinase inhibitors. In the case of p21, TGF-β treatment leads to an increase in p21 mRNA. This increase in p21 mRNA is partly due to transcriptional activation of the p21 promoter by TGF-β. The region in the p21 promoter responsive to TGF-β signaling consists of a 10-base-pair sequence that binds transcription factors such as transcription factors Sp-1 and Sp-3; it is required for activation of the p21 promoter by TGF-β. In addition, this sequence is sufficient to drive TGF-β-mediated transcription from a previously nonresponsive promoter (Hocevar and Howe, 1998).

[0057] TGF-β also inhibits cell-cycle progression in many cell types. The TGF-β-induced cell-cycle arrest has been partially attributed to the regulatory effects of TGF-β on both the levels and the activities of the G1 cyclins and their cyclin-dependent kinase (cdk) partners. Furthermore, it has been postulated that TGF-β inhibits cell-cycle progression by blocking the late G1 activation of the cdks, thereby preventing pRb phosphorylation and S phase entry (Hocevar and Howe, 1998).

[0058] In $Rb^{+/+}$ and $Rb^{-/-}$ primary mouse embryo fibroblasts, TGF-β inhibited cdk4-associated kinase activity. However, whereas cdk2-associated kinase activity was completely inhibited by TGF-β in the wild-type cells, it was reduced only slightly in the Rb mutant cells. Moreover, at high cell density, the growth-inhibitory effects of TGF-β were no longer observed in the $Rb^{-/-}$ cells. On the contrary, TGF-β treatment promoted the growth of these mutant

fibroblasts (Herrera *et al.*, 1996; and Zhang and Jacobberger, 1996). Thus, under certain cellular-growth conditions, elimination of pRb transforms the growth-inhibitory effects of TGF-β into growth-stimulatory effects. These observations could help explain why TGF-β is often found to enhance tumorigenicity *in vivo,* and why inactivation of the Rb gene leads to tumorigenesis.

[0059] TGF-β was identified by its ability to cause phenotypic transformation of rat fibroblasts. Later, experiments showed that TGF-β plays an important role in the deposition of extracellular matrix and the development of fibrosis. To evaluate the role of TGF-β-1 in the pathogenesis of fibrosis, Clouthier *et al.* (1997) used a transgenic approach. They targeted the expression of a constitutively-active TGF-β-1 molecule to liver, kidney, and white and brown adipose tissue, using the regulatory sequences of the rat phosphoenolpyruvate carboxykinase gene. In multiple lines, targeted expression of the transgene caused severe fibrotic disease. Fibrosis of the liver occurred with varying degrees of severity, depending upon the level of expression of the TGF-β-1 gene. Overexpression of the transgene in kidney also resulted in fibrosis and glomerular disease, eventually leading to complete loss of renal function. Severe obstructive uropathy (hydronephrosis) was also observed in a number of animals. Expression in adipose tissue resulted in a dramatic reduction in total body white adipose tissue, and a marked, though less severe, reduction in brown adipose tissue, producing a lipodystrophy-like syndrome. Introduction of the transgene into the ob/ob background suppressed the obesity characteristic of this mutation; however, transgenic mutant mice developed severe hepatomegaly and splenomegaly. Clouthier *et al.* (1997) noted that the family of rare conditions known collectively as the lipodystrophies are accompanied in almost all forms by other abnormalities, including fatty liver and cardiomegaly. Metabolic and endocrine abnormalities include either mild or severe insulin resistance, hypertriglyceridemia, and a hypermetabolic state.

[0060] Using quantitative PCR in 15 cases of Duchenne muscular dystrophy (DMD), 13 cases of Becker muscular dystrophy, 11 spinal muscular atrophy patients, and 16 controls, Bernasconi *et al.* (1995) found that TGF-β-1 expression, as measured by mRNA, was greater in DMD patients than in controls. Furthermore, fibrosis was significantly more prominent in DMD than in controls. The proportion of connective tissue biopsies increased progressively with age in DMD patients, with TGF-β-1 levels peaking at 2 and 6 years of age. Bernasconi *et al.* (1995) concluded that expression of TGF-β-1 in the early stages of DMD may be critical in initiating muscle fibrosis, and suggested that an antifibrosis treatment might slow progression of the disease, thereby increasing the utility of gene therapy.

[0061] Choi *et al.* (1996) showed that, when topically applied, oligodeoxynucleotides complementary to TGF-β-1 mRNA significantly reduced scarring. Liu *et al.* (1995) showed that TGF-β can induce collagen formation, and mRNA expression of type I and type IV collagen, without affecting cell proliferation in cultures of human embryonic lung fibroblasts.

[0062] TGF-β also plays a role in the coagulation process. TGF-β, which is present in the blood (Grainger *et al.*, 1995a), is released from blood clot during dissolution by plasmin (Grainger *et al.*, 1995b). TGF-β induces the plasminogen activator (Arnoletti *et al.*, 1995), tissue factor, and PAI-1 inhibitor, leading to the development of a hypofibrinolytic state (Dennler *et al.*, 1998; Dong *et al.*, 1996; and Samad *et al.*, 1998).

(b) Methods and Results

[0063] P19 totipotent embryonal carcinoma cells were transfected with the artificial construct, pSBE(Smad binding element)-Lux. In this construct, the luciferase gene is under the control of the SBE – an element in the promoter of TGF-β-responsive genes which is activated by direct binding of the TGF-β-induced transcriptional complex (Luigi *et al.*, 1998). The luciferase activity of cells transfected with the construct was assessed in the presence of TGF-β and

varying amounts of ebaf. Ebaf, in a dose-dependent fashion, inhibited the activity of the reporter (Figure 3A). This inhibitory activity, however, could be overridden by increasing the concentration of TGF-β, thereby indicating that the extent of inhibition depends on a balanced amount of TGF-β and ebaf (Figure 3B).

[0064] The inventors next tested the effect of ebaf on TGF-β-mediated regulation of the activity of reporters of cell cycle factors p21 and Cdc25. In untransformed epithelial cells, the G1 cell cycle events which are mediated by TGF-β include up-regulation of p21(cip1) and reduction of Cdc25 (Datto *et al.*, 1995; Hartsough, 1997; and Iavarone and Massagué, 1997). P19 cells were transfected with p21-Lux and pCdc25-Lux constructs, and the reporter activity was assessed in the presence of TGF-β and ebaf. TGF-β increased p21 reporter activity, and decreased Cdc25 reporter activity (Figures 3C-D). While ebaf did not have any discernible effect on its own, it significantly inhibited the reporter activity regulated by TGF-β (Figures 3C and 3D). Whereas Cdc25 reporter activity was reduced almost to 50%, the activity of the p21 promoter was reduced more than four-fold. These findings suggest that ebaf inhibits several well-known functions of TGF-β that control cell proliferation in epithelial cells.

[0065] To determine whether ebaf activities are primarily confined to TGF-β-mediated control of cell cycle factors, or whether they also target other known functions of TGF-β, the inventors further tested the effect of ebaf on TGF-β-mediated connective tissue growth factor (CTGF) promoter activity. TGF-β leads to fibrogenesis by activating the transcription of CTGF, a cytokine which induces collagen synthesis by fibroblasts (Frazier *et al.*, 1996; Grotendorst, 1997; and Duncan *et al.*, 1999). Ebaf significantly reduced the activity of the reporter induced by 5 ng/ml of TGF-β and 15 ng/ml (not shown) of TGF-β, and brought the reporter activity to basal levels (Figure 3E). These findings show that ebaf is a broad-range inhibitor of TGF-β activities.

[0066] Biological signaling of TGF-β involves heterodimerization of Smad2/3 with Smad4, and subsequent nuclear translocation of these proteins

(Younai *et al.*, 1994; Lin *et al.*, 1995; Zhang *et al.*, 1995; Choi *et al.*, 1996; Yoshida and Hayashi, 1996; Coker *et al.*, 1997; Heldin *et al.*, 1997; Liu *et al.*, 1997; Derynck *et al.*, 1998; Howell *et al.*, 1999; Faure *et al.*, 2000; and Weinstein *et al.*, 2000). Therefore, the inventors reasoned that the inhibitory effect of ebaf could be exerted on TGF-β-mediated heterodimerization of Smads, and subsequent nuclear translocation of these heteromeric complexes.

[0067] As a first step towards elucidation of such a role, P19 cells were treated with TGF-β, in the presence and absence of ebaf. After 1 h of treatment, the cytosol and nuclear lysates of these cells were subjected to Western blotting for Smad2, Smad4, and Smad 5. In comparison with the control cells, TGF-β led to the accumulation of Smad2 and Smad4 in the nuclei of treated cells (Figure 4A; arrows). TGF-β did not have any effect on nuclear translocation of Smad5, which is an intracellular mediator of the BMP signaling pathway (Kawabata *et al.*, 1998). While ebaf did not change the amount of Smads in the cytosol or the nuclei of the treated cells, it did inhibit the TGF-β-induced nuclear translocation of both Smad2 and Smad4 (Figure 4A). Immunolocalization of Smad4 in P19 cells treated with TGF-β or ebaf showed nuclear accumulation of Smad4 by TGF-β treatment. In contrast, ebaf did not increase the amount of nuclear Smad4 on its own; rather, it inhibited the TGF-β-induced Smad4 nuclear accumulation (Figure 4B). These findings show that ebaf prevents the TGF-β-mediated nuclear accumulation of the Smad2/4 complex required for gene transcriptional activity.

[0068] In light of these findings, the inventors assessed the extent to which ebaf can inhibit the TGF-β-mediated heterodimerization of Smad2 with Smad4. Smad2 was immunoprecipitated from the nuclear fraction of P19 cells which had been treated with TGF-β and/or ebaf. The immunoprecipitates were subjected to Western blotting for Smad4 (Figure 5). As expected, in the TGF-β-treated cells, Smad4 was present in the Smad2 immunoprecipitate, showing that it had heterodimerized with Smad2 (Figure 5). Ebaf had no effect on this event, but did prevent the TGF-β heterodimerization of Smad4 with Smad2 (Figure 5).

Treatment of cells with TGF-β or ebaf did not lead to any detectable changes in the total amount of Smad2 or Smad4 (Figure 5).

[0069] Formation of the Smad heteromeric complexes requires phosphorylation of the Smad2/3 proteins by the serine kinase activity of TGF-β receptor type I, after binding of TGF-β to its receptors (Younai *et al.*, 1994; Lin *et al.*, 1995; Zhang *et al.*, 1995 Choi *et al.*, 1996; Yoshida and Hayashi, 1996; Coker *et al.*, 1997; Heldin *et al.*, 1997; Liu *et al.*, 1997; Derynck *et al.*, 1998; Howell *et al.*, 1999; Faure *et al.*, 2000; and Weinstein *et al.*, 2000). To determine whether ebaf exerts its function by interfering with this essential step, the inventors next examined the effect of ebaf on TGF-β-mediated Smad2 phosphorylation (Figure 6). P19 cells were treated with TGF-β, ebaf, or both, in the presence of [^{32}P]-orthophosphate, in order to label phosphorylated proteins. Smad2 proteins in the lysates were immunoprecipitated. Immunoprecipitates were subjected to SDS-gel electrophoresis and Western blotting, followed by autoradiography. As expected, TGF-β led to the phosphorylation of Smad2. Although, ebaf did not alter the phosphorylation of Smad2, it reduced the TGF-β-mediated Smad2 phosphorylation by 60% (Figure 6).

[0070] To exert its function, TGF-β oligomerizes type I and type II receptors on the cell surface (Wrana *et al.*, 1992; and Bassing *et al.*, 1994). In the complexes which are formed, type I receptors get phosphorylated by the constitutively-active type II receptors (Wrana *et al.*, 1994). To ascertain whether ebaf has inhibitory activity because it interferes with the binding of TGF-β to its receptors and/or the oligomerization of the receptors, or whether ebaf's inhibitory activity is independent of these events, the inventors first analyzed the effect of ebaf on gene transcription driven by a constitutively-active form of TGF-β receptor type I. The receptor was transfected along with pSBE-Lux construct into P19 cells, and ebaf's effect on reporter activity was assessed. Ebaf, in a dose-dependent fashion, inhibited the activity of the reporter induced by the receptor (Figure 7A). Moreover, ebaf inhibited by 80% the phosphorylation of Smad2 by the receptor (Figure 7B).

[0071] The inventors further analyzed the interaction of ebaf with the TGF-β receptor as a possible mechanism for the inhibitory activity of ebaf. Radioiodinated TGF-β was cross-linked to the TGF-β receptors in the presence of varying amounts of ebaf. The receptor cross-linked to TGF-β was immunoprecipitated by an antibody to TGF-β receptor type I. The immunoprecipitates then were subjected to gel electrophoresis, followed by autoradiographic assessment of the amount of radioactivity. Ebaf, at its biologically-effective doses, failed to inhibit the binding of TGF-β to receptor type I (Figure 8A); it also failed to inhibit the amount of Smad2 bound to the receptor immunoprecipitated by an antibody to Smad2 (Figure 8B). These findings show that inhibition by ebaf of TGF-β signaling does not involve competition between TGF-β and ebaf for binding to the type I receptor.

[0072] One possibility for ebaf's inhibition of TGF-β-mediated activities is induction of expression of inhibitory Smad proteins (anti-Smads). Two Smad proteins, Smad6 and Smad7, inhibit the actions of TGF-β intracellularly. Smad7 interferes with TGF-β signaling by interaction with, and inhibition of phosphorylation of, receptor-bound Smads (Hayashi *et al.*, 1997; and Nakao *et al.*, 1997). Overexpression of Smad7 has been shown to be responsible for the antagonistic effect of IFN-γ on TGF-β-mediated cellular functions (Ulloa *et al.*, 1999). Smad6 forms stable associations with type I receptors, and interferes with the phosphorylation of Smad2 and its heterodimerization with Smad4; however, Smad6 does not inhibit the phosphorylation of Smad3 (Imamura *et al.*, 1997; Nakayama *et al.*, 1998; and Hata *et al.*, 1998).

[0073] To determine whether the inhibitory activity exhibited by ebaf involves synthesis of Smad7, P19 cells were treated with ebaf for various periods of time. The cell lysates then were subjected to Western blot analysis for Smad7 (Figure 9A). Ebaf did not induce any change in the total amount of Smad7. Similarly, treatment of cells with ebaf failed to induce any change in the amount of Smad6 (data not shown). To determine whether the inhibitory activity of ebaf requires synthesis of any other protein, the phosphorylation of Smad2 was

analyzed, in the presence and absence of ebaf, in P19 cells that had been treated with cyclohexamide and transfected with constitutively-active TGF-β receptor type I. In the presence of cyclohexamide, ebaf inhibited Smad2 phosphorylation by the active TGF-β receptor type I (Figure 9B). Taken together, these findings show that inhibition of the activity of TGF-β by ebaf does not require synthesis of Smad6, Smad7, or any other protein.

[0074] Accumulation of Smads is induced by receptor-mediated phosphorylation at their carboxy termini, and can be inhibited by MAP kinase-mediated phosphorylation at their central regions. For this reason, the effect of ebaf on MAP kinase was assessed for the potential to restrict Smad activation. A conditioned medium of cells transfected with lefty-A (ebaf) and lefty-B was able to activate the MAPK pathway. A conditioned medium of cells transfected with the GGKG *lefty-A* (*ebaf*) mutant cDNA, which led to loss of the 34-kD form of the lefty-A (ebaf) protein into the culture medium, also exhibited this activity (Figure 10, upper panel). However, a conditioned medium of cells transfected with the GHGR *lefty-A* (*ebaf*) mutant, which led to loss of the 28-kD protein, did not show this activity.

[0075] To demonstrate that this activity was due to ebaf protein in the culture medium, the effect of the medium was examined in the presence of varying amounts of affinity-purified rabbit polyclonal antibody specific to ebaf (Figure 10, middle panel). The activity of the conditioned medium was inhibited, in a dose-dependent fashion, in the presence of the antibody. To directly show that this activity was mediated by the 28-kD form of the ebaf protein, the activity of the 26-kD recombinant *E. coli* ebaf protein, which corresponded to the non-glycosylated 28-kD ebaf, was tested (Figure 10, lower panel). The 26-kD recombinant protein induced MAPK activation in a dose-dependent fashion. Activation of MAPK by ebaf was validated by Western blot analysis (Figure 11). Recombinant *E. coli* ebaf induced the phosphorylation of the p42/44-kD proteins in 7 min; this phosphorylation reached a maximum within 15 min of incubation with ebaf.

[0076] The inventors also tested the effect on the c-Jun N-terminal kinase (JNK) pathway of culture media containing cells transfected with lefty-A (ebaf), lefty-B, and the 26-kD recombinant *E. coli*-produced ebaf protein. However, no effect on the JNK pathway was observed.

2. Ebaf Leads to Fibroblast Death

(a) Introduction

[0077] Transforming growth factor beta (TGF-β) protects fibroblasts from apoptosis-inducing signals and promotes fibroblast proliferation (Tredget *et al.*, 2000; and Fine and Goldstein, 1987). TGF-β inhibits apoptosis by causing cellular production of a distinct set of proteins. Specifically, the treatment of NRK 536 fibroblasts with TGF-β caused a reversible transformed phenotype (Vossbeck *et al.*, 1995). A 15-kD membrane adhesion protein, called TGF-β induced factor 2 (TIF2), has been identified as one of the proteins induced by TGF-β which mediates this transforming ability. This factor is able to provide resistance to TGF-β induced apoptosis (Carey and Chang, 1998). Fibrosis is a process that involves undesirable proliferation of fibroblasts. TGF-β in such lesions promotes fibrosis by promoting proliferation and inhibiting fibroblast apoptosis. Identification of a factor that counteracts this action of TGF-β could be useful therapeutically. The inventors have found that ebaf leads to fibroblast death. This specificity renders ebaf particularly useful in the treatment of fibrotic disorders.

(b) Materials and Methods

[0078] Retroviral Vector Expression Plasmid Construction: LX-Ebaf-IRES-eGFP (LEIG): Plasmid LX-ebaf was double digested with Sph I and Xho I to generate a 6155-bp fragment. Oligonucleotide primers NS204 (5'aaagatatcgcatgccctctccctcccc ccccccctaacg3') and NS205 (5'tttgatatcctcgagttacttgtacagctcgtccatgcc3') were used as PCR primers with plasmid pIRES-eGFP (Clontech) to generate a 1338 bp Sph I/Xho I IRES-eGFP

fragment which was cloned into the 6155-bp LX-ebaf fragment to generate plasmid LEIG. This retroviral vector plasmid can be used to generate retroviral vector particles for transduction of various cells and cell lines when transfected into retroviral packaging cell lines such as PA317 and GP+E86.

[0079] Mammalian Expression Plasmid Construction: The sense and anti-sense orientations of the ebaf cDNA were constructed using plasmid pAdCMV5 (Quantum Biotechnologies Inc., Montreal, Canada) in which ebaf gene expression is regulated by the cytomegalovirus immediate early promoter. A 1.2-kb BamHI/AflIII *ebaf* cDNA fragment containing minimal 5' and 3' untranslated regions was isolated from plasmid pBluescript2SK-ebaf, filled with T4 DNA polymerase, and cloned into PmeI-digested pAdCMV5. Restriction mapping and flanking DNA sequencing confirmed the orientation of the resulting ebaf expression plasmids.

[0080] MTT assay: The MTT assay was performed as per manufacturer's instructions (SIGMA BioSciences):

1. CCD19Lu cells that were treated in duplicate wells of 6-well dishes were washed twice with PBS.
2. 450 µl of DMEM supplemented with 10% heat-inactivated FBS was added to each well and to two blank wells that served as medium-only controls.
3. 50 µl of MTT Solution (SIGMA BioSciences, cat. # M0283) was added to each well, and cells were incubated for 4 h at 37°C in 5% CO_2.
4. 500 µl of MTT Solvent (SIGMA BioSciences, cat. # M0408) was added to the wells and repeatedly triturated with the cells using a pipette to lyse the cells and dissolve the formazan crystals.
5. Aliquots of the cell lysates were transferred in triplicate to a 96-well microtiter plate.
6. An ELISA plate reader was used to measure the amount of formazan crystals generated from living cells. The test wavelength was 540 nm, and the reference wavelength was 690 nm.

(c) Results

[0081] The inventors used a mammalian expression plasmid to stably express ebaf in both an epithelial (293) and a fibroblastic cell line (NIH3T3). Both cell lines produced ebaf. However, the NIH3T3 cell line lost its ability to synthesize ebaf within weeks after transfection, whereas the 293 cells were capable of producing ebaf for a year and a half post-transfection while maintained under G418 selective pressure for plasmid maintenance. These findings suggest that the expression of ebaf is detrimental to NIH3T3 cells, and that the ebaf-positive cells are lost.

[0082] The inventors also transfected 293 cells and a fibroblastic cell line, PA137, with a retroviral vector expression plasmid (LEIG) capable of expressing both ebaf and a green fluorescent protein (GFP). Another retroviral vector expression plasmid, expressing only GFP (LG), was used as a control. The 293 cells expressed both ebaf and GFP upon transfection with LEIG, and expressed only GFP upon transfection with LG. The PA137 cells that were transfected with LG expressed GFP. Interestingly, the PA137 cells that were transfected with LEIG resulted in large amounts of cell death, with little or no GFP expression, suggesting toxicity of ebaf overexpression in these cells.

[0083] The inventors then proceeded to treat the human fibroblastic cell line, CCD19Lu, with 24-hour-conditioned medium collected from both 293 cells and 293 cells that were stably transfected with LEIG and known to express both ebaf and GFP. The conditioned medium was replaced approximately every two days with fresh conditioned medium on the CCD19Lu cells. The CCD19Lu cells treated with 293 conditioned medium (without ebaf and GFP) continued to grow and proliferate normally. In contrast, the CCD19Lu cells treated with conditioned medium from the 293 cells expressing both ebaf and GFP resulted in a large amount of cell death starting on the second day of treatment.

[0084] Initially, the CCD19Lu cells developed an abnormal morphology, including loss of cytoplasm and development of a spindly appearance, followed by rounding and detachment from the dish (Figure 12). An MTT assay (a

measure of live cells) was performed on the treated CCD19Lu cells at day 9 following initial treatment (Figure 13). At this time, microscopic examination of the CCD19Lu cells indicated that the cells treated with 293 conditioned medium were healthy, confluent cultures of cells. Conversely, the CCD19Lu cells treated with the 293 medium containing ebaf were either dead or appeared to be undergoing cell death. Results of the MTT assay indicate that there is at least a 15-fold decrease in the number of live cells in the ebaf containing medium compared to the control containing only 293 conditioned medium.

3. Ebaf Leads to Regression of Tumors

[0085] The inventors tested the effect of ebaf on growth of fibroblastic cells by injecting into nu/nu mice GP+E86 cells transduced with a retroviral vector expressing green fluorescent protein (LG) or green fluorescent protein (GFP) and ebaf (LEIG). Cells were injected both subcutaneously and intraperitoneally. As shown in Figure 14, after 3 wk, the size and volume of the subcutaneous and peritoneal tumors were significantly smaller in the LG-injected mice, as compared with the animals injected with LEIG cells.

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[0086] All publications mentioned hereinabove are hereby incorporated in their entireties. While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art, from a reading of the disclosure, that various changes in form and detail can be made without departing from the true scope of the invention in the appended claims.

What is claimed is:

1. A method for inhibiting activity of TGF-β, comprising contacting tissue expressing TGF-β with an amount of ebaf or an ebaf analogue effective to inhibit the activity of TGF-β.

2. The method of Claim 1, wherein tissue expressing TGF-β is contacted with ebaf or an ebaf analogue by introducing to the tissue ebaf protein or ebaf analogue protein.

3. The method of Claim 1, wherein tissue expressing TGF-β is contacted with ebaf or an ebaf analogue by introducing to the tissue a nucleic acid encoding ebaf or the ebaf analogue, in a manner permitting expression of ebaf or the ebaf analogue.

4. The method of Claim 3, wherein the nucleic acid is introduced by a method selected from the group consisting of electroporation, DEAE Dextran transfection, calcium phosphate transfection, cationic liposome fusion, protoplast fusion, creation of an *in vivo* electrical field, DNA-coated microprojectile bombardment, injection with recombinant replication-defective viruses, homologous recombination, gene therapy, viral vectors, and naked DNA transfer.

5. The method of Claim 1, wherein the contacting is effected *in vivo*.

6. The method of Claim 5, wherein the contacting is effected *in vivo* in a mammal.

7. The method of Claim 6, wherein the mammal is a human.

8. The method of Claim 7, wherein the human has a condition associated with overactivity of TGF-β.

9. The method of Claim 8, wherein the condition is fibrosis.

10. The method of Claim 9, wherein the fibrosis is a scar, a keloid, cirrhosis, Asherman's syndrome, Meigs' syndrome, a muscular dystrophy, an autoimmune disorder, post-surgical fibrosis, or primary pulmonary fibrosis.

11. The method of Claim 10, wherein the scar results from a burn, radiation, a chemical, or a myocardial infarct.

12. The method of Claim 10, wherein the muscular dystrophy is Duchenne muscular dystrophy.

13. The method of Claim 10, wherein the autoimmune disorder is scleroderma.

14. The method of Claim 10, wherein the primary pulmonary fibrosis is Hamman Rich Syndrome or retroperitoneal fibrosis.

15. The method of Claim 8, wherein the condition is a defect in cell proliferation.

16. The method of Claim 15, wherein the defect in cell proliferation is hyperplasia or neoplasia.

17. The method of Claim 8, wherein the condition is a coagulation defect.

18. The method of Claim 17, wherein the coagulation defect is menstrual bleeding, abnormal uterine bleeding, coagulopathy, or toxemia of pregnancy.

19. The method of Claim 7, wherein tissue expressing TGF-β is contacted with ebaf or an ebaf analogue by introducing to the tissue ebaf protein or ebaf analogue protein.

20. The method of Claim 7, wherein tissue expressing TGF-β is contacted with ebaf or an ebaf analogue by introducing to the tissue a nucleic acid encoding ebaf or the ebaf analogue, in a manner permitting expression of ebaf or the ebaf analogue.

21. The method of Claim 20, wherein the nucleic acid is introduced by a method selected from the group consisting of electroporation, DEAE Dextran transfection, calcium phosphate transfection, cationic liposome fusion, protoplast fusion, creation of an *in vivo* electrical field, DNA-coated microprojectile bombardment, injection with recombinant replication-defective viruses, homologous recombination, gene therapy, viral vectors, and naked DNA transfer.

22. A method for inhibiting activity of TGF-β, comprising contacting tissue expressing TGF-β with a modulator of ebaf expression, or a modulator of expression of an ebaf analogue, in an amount effective to induce or enhance expression of ebaf or the ebaf analogue and inhibit the activity of TGF-β.

23. The method of Claim 22, wherein the modulator of expression is retinoic acid, estrogen, or progesterone.

24. A method for treating fibrosis in a subject in need of treatment, comprising administering to the subject an amount of ebaf or an ebaf analogue effective to treat the fibrosis.

25. The method of Claim 24, wherein the fibrosis is a scar, a keloid, cirrhosis, Asherman's syndrome, Meigs' syndrome, a muscular dystrophy, an autoimmune disorder, post-surgical fibrosis, or primary pulmonary fibrosis.

26. The method of Claim 25, wherein the scar results from a burn, radiation, a chemical, or a myocardial infarct.

27. The method of Claim 25, wherein the muscular dystrophy is Duchenne muscular dystrophy.

28. The method of Claim 25, wherein the autoimmune disorder is scleroderma.

29. The method of Claim 25, wherein the primary pulmonary fibrosis is Hamman Rich Syndrome or retroperitoneal fibrosis.

30. A method for treating a defect in cell proliferation in a subject in need of treatment, comprising administering to the subject an amount of ebaf or an ebaf analogue effective to treat the defect in cell proliferation.

31. The method of Claim 30, wherein the defect in cell proliferation is hyperplasia or neoplasia.

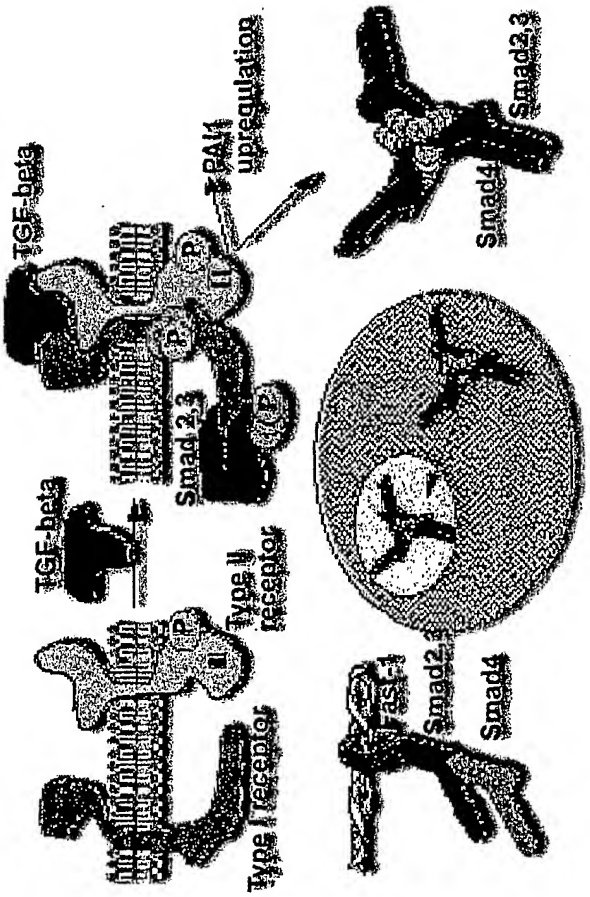
TGF-beta
TGF-beta
Type II receptor
Type I receptor
Smad 2,3
PAI-1 upregulation
Smad4
Smad2,3
Fast-1
Smad2,3
Smad4

Figure 1

cDNA sequence

```
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GCCCTGACCGAGGAGCAGCTCCTGGGCAGCCTGCTGCGGCAGCTGCAGCTCAG
CGAGGTGCCCGTACTGGACAGGGCCGACATGGAGAAGCTGGTCATCCCCGCCC
ACGTGAGGGCCCAGTATGTAGTCCTGCTGCGGCGCAGCCACGGGGACCGCTCC
CGCGGAAAGAGGTTCAGCCAGAGCTTCCGAGAGGTGGCCGGCAGGTTCCTGGC
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CCAACAGCGAGCTGGTGCAGGCCGTGCTGCGGCTCTTCCAGGAGCCGGTCCCC
AAGGCCGCGCTGCACAGGCACGGGCGGCTGTCCCCGCGCAGCGCCCAGGCCC
GGGTGACCGTCGAGTGGCTGCGCGTCCGCGACGACGGCTCCAACCGCACCTCC
CTCATCGACTCCAGGCTGGTGTCCGTCCACGAGAGCGGCTGGAAGGCCTTCGA
CGTGACCGAGGCCGTGAACTTCTGGCAGCAGCTGAGCCGGCCCCGGCAGCCGC
TGCTGCTACAGGTGTCGGTGCAGAGGGAGCATCTGGGCCCGCTGGCGTCCGGC
GCCCACAAGCTGGTCCGCTTTGCCTCGCAGGGGGCGCCAGCCGGGCTTGGGGA
GCCCCAGCTGGAGCTGCACACCCTGGACCTCAGGGACTATGGAGCTCAGGGCG
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Amino Acid Sequence

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GAHKLVRFASQGAPAGLGEPQLELHTLDLRDYGAQGDCDPEAPMTEGTRCCRQEMYIDLQGMKWAKNWVLE
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Figure 2

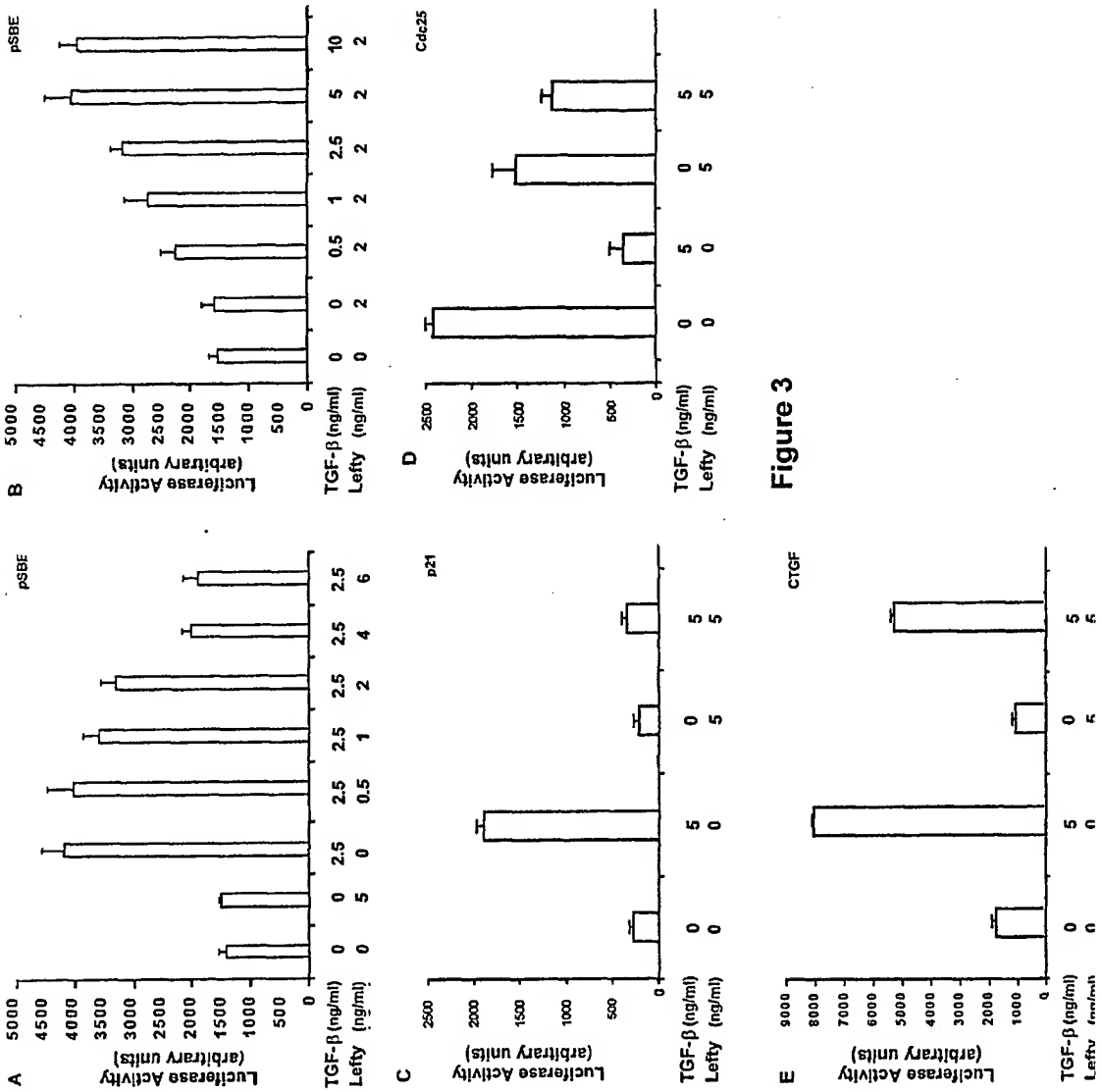

A
pSBE
Luciferase Activity (arbitrary units)
TGF-β (ng/ml)
Lefty (ng/ml)
B
pSBE
C
p21
D
Cdc25
E
CTGF

Figure 3

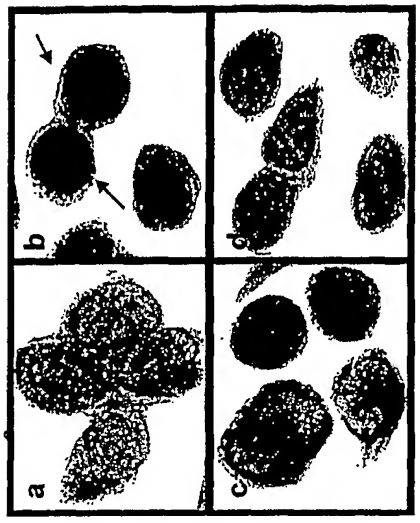


Figure 4B

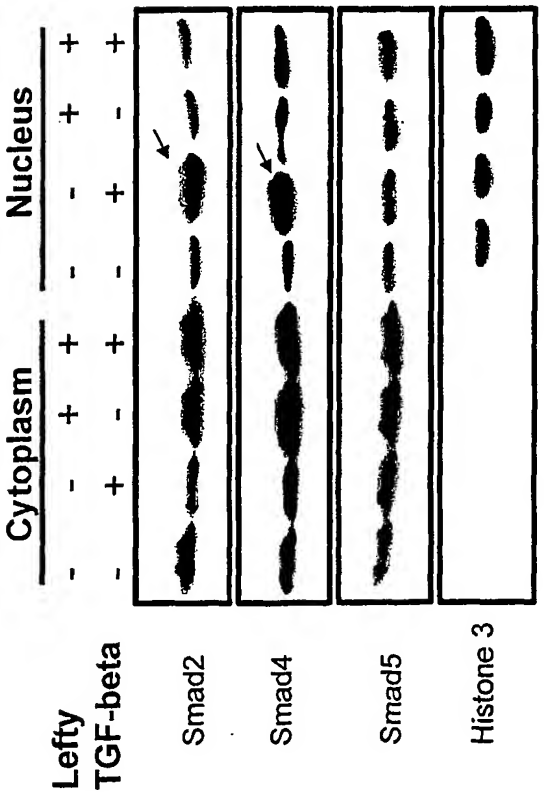


Figure 4A

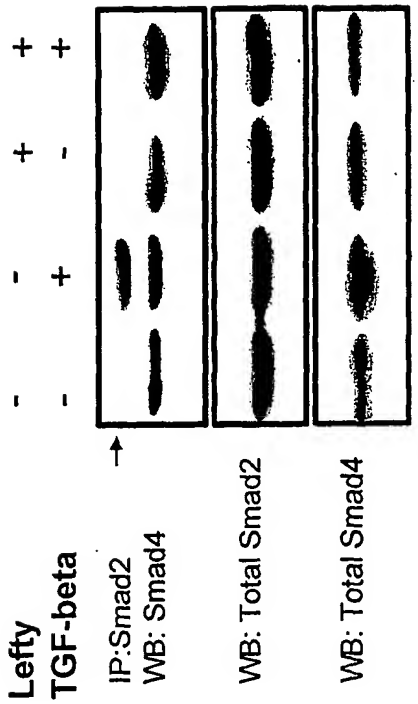

Lefty
TGF-beta
- - + - + +
- + - + - +
IP:Smad2
WB: Smad4
WB: Total Smad2
WB: Total Smad4

Figure 5

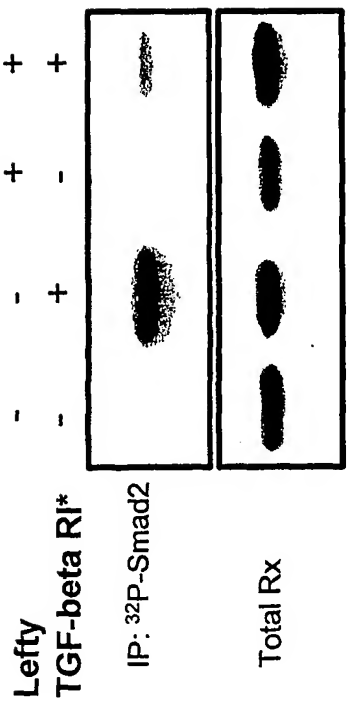


Figure 6

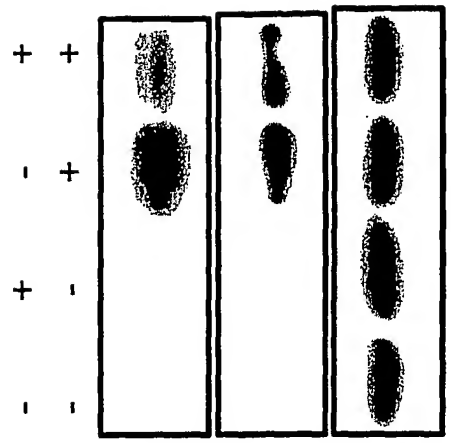


Figure 7B

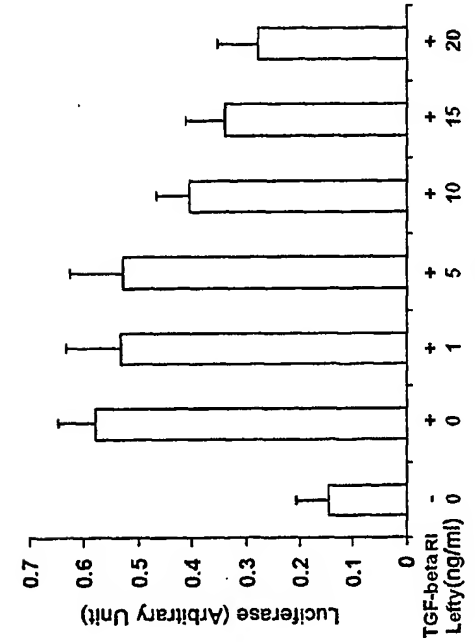


Figure 7A

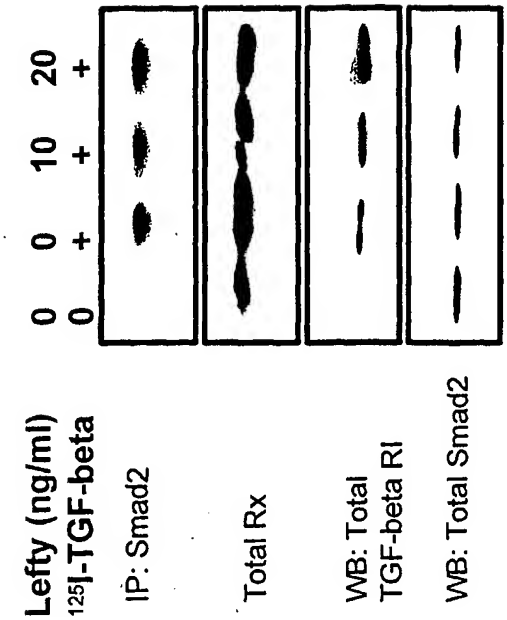


Figure 8B

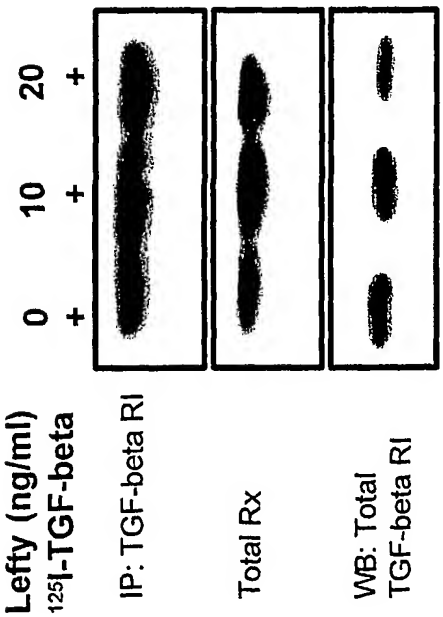


Figure 8A

Figure 9B

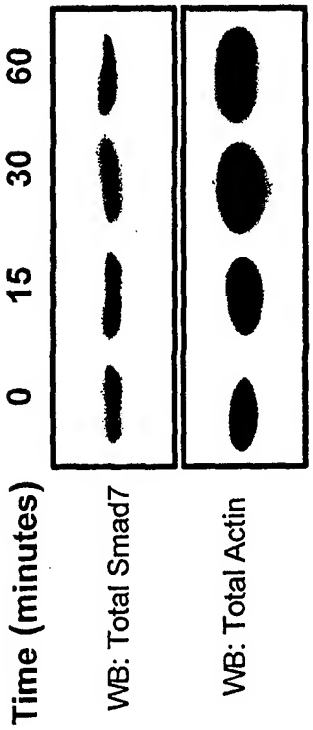
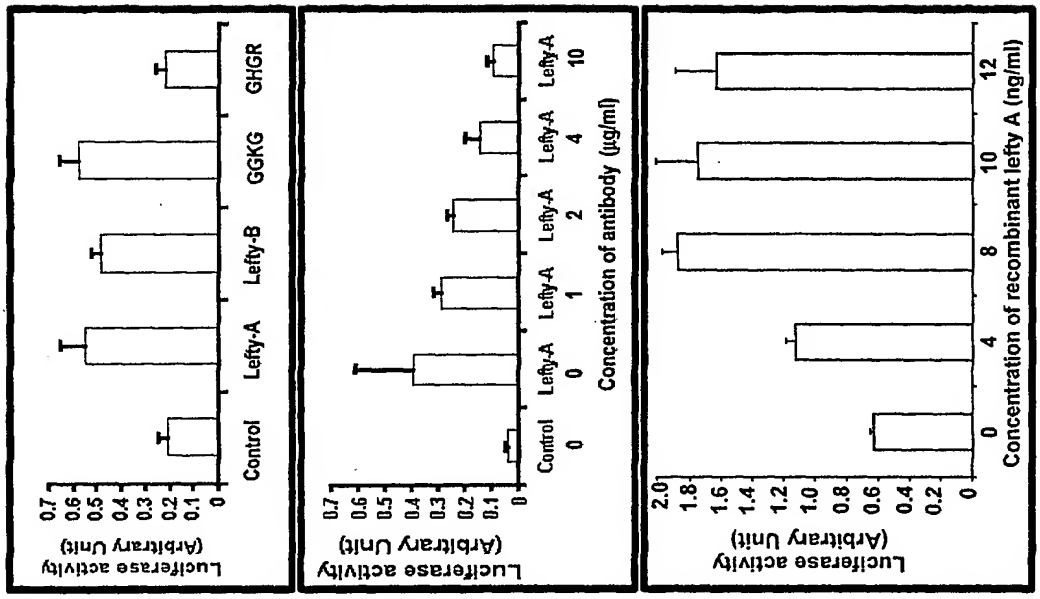


Figure 9A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0.6
0.5
0.4
0.3
0.2
0.1
0
Control
Lefty-A
Lefty-B
GGKG
GHGR
Luciferase activity
(Arbitrary Unit)
Control
Lefty-A
Lefty-A
Lefty-A
Lefty-A
Lefty-A
0
0
1
2
4
10
Concentration of antibody (µg/ml)
Luciferase activity
(Arbitrary Unit)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
4
8
10
12
Concentration of recombinant lefty A (ng/ml)

Figure 10

	lefty-A (15 ng/ml)					EGF (15 ng/ml)
Duration of incubation (minutes)	0	7	15	30	60	15
Antibody for WB, Upper panel	Phosphorylated p42/44 kD proteins					
Antibody for WB, Lower Panel	Total p42/44 kD proteins					
Lane #	1	2	3	4	5	6

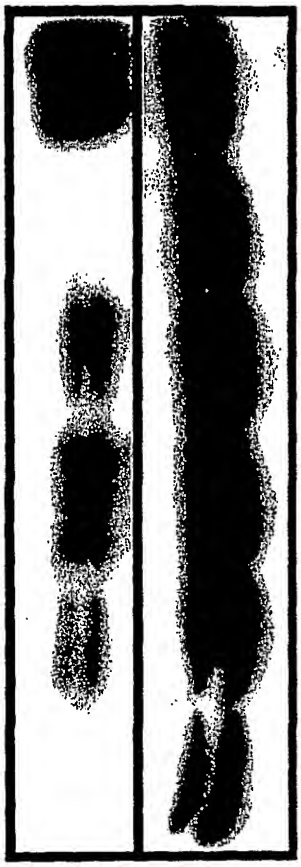

Figure 11

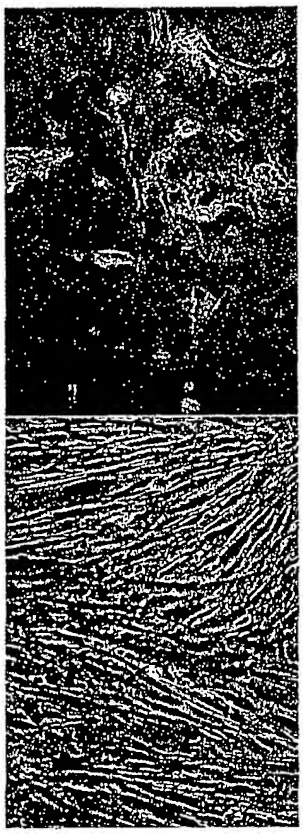

Figure 12

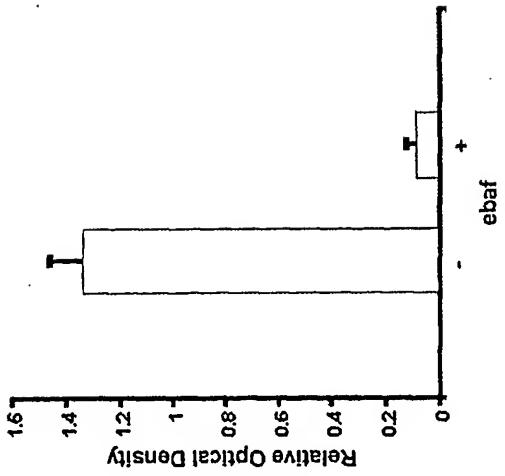

Figure 13

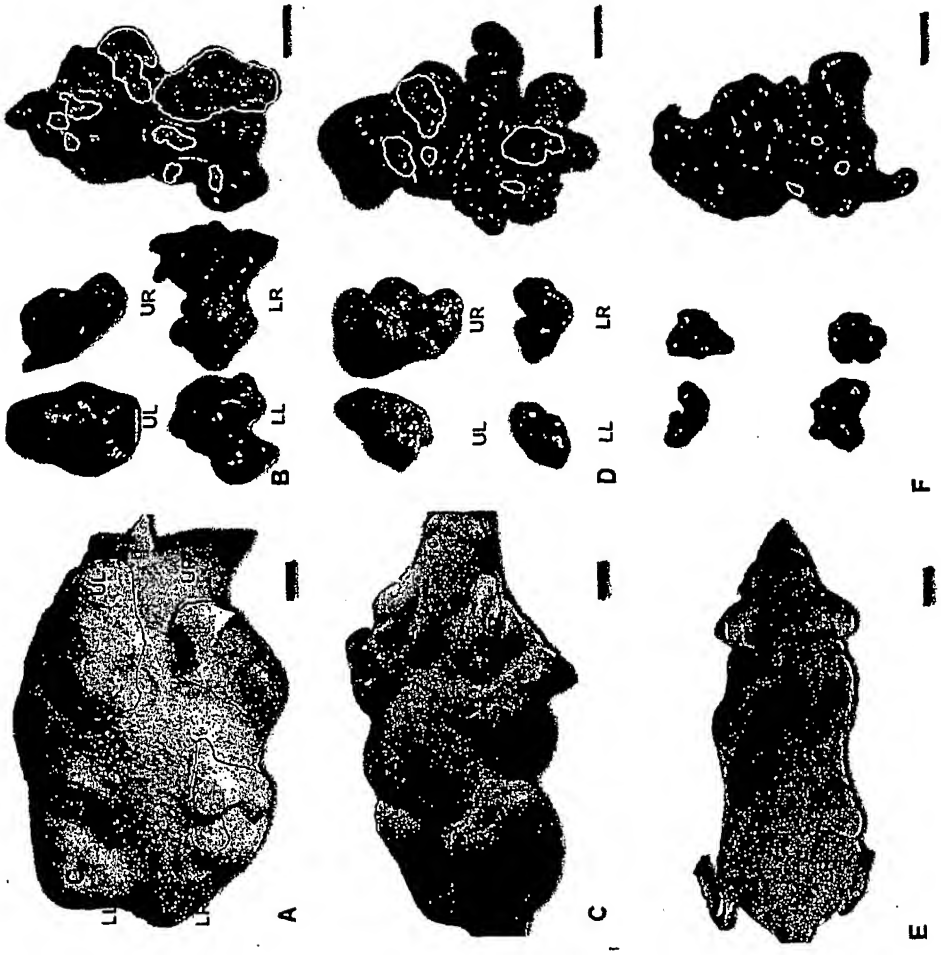

Figure 14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/30872

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C 12 Q 1/68; G 01 N 33/574; C 07 K 1/00, 14/00, 17/00; A 61 K 38/00; A 01 N 37/18
US CL : 435/6, 7.23; 530/350; 514/12, 2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/6, 7.23; 530/350; 514/12, 2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,916,751 A (TABIBZADEH et al.) 29 June 1999 (29.06.1999), see entire document.	1-31
A	US 5,821,227 A (DENNIS et al.) 13 October 1998 (13.10.1998), see entire document.	1-31
A	TABIBZADEH, S. Dysregulated Expression of ebaf a Novel Molecular Defect in the Endometria of Patients with Infertility, Journal of Clinical Endocrinology and Metabolism. August 2000, Vol.85 no.7, pages 2526-2536, see entire document.	1-31

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
18 November 2001 (18.11.2001)

Date of mailing of the international search report
05 FEB 2002

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703)305-3230

Authorized officer
Konstantina Katcheves

Telephone No. (703) 305-3388

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/30872

Continuation of B. FIELDS SEARCHED Item 3:
EAST, CAPLUS, MEDLINE, BIOSIS
search terms: TGF.beta., inhibit, down-regulate, endothelial bleeding associated factor, TGFB-4

TGF-β antagonists: Why suppress a tumor suppressor?

Commentary

See related articles, pages 1551–1559 and pages 1607–1615.

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Phone: (415) 514-0215; Fax: (415) 502-6779; E-mail: rakhurst@cc.ucsf.edu.

J. Clin. Invest. **109**:1533–1536 (2002). doi:10.1172/JCI200215970.

Tumor metastasis is the major determinant of cancer patient survival. This ultimate phase in tumorigenesis depends on the ability of a tumor cell to invade the stroma, migrate in and out of blood or lymphatic vessels, and survive and re-establish itself at a secondary site. A large number of papers have provided strong evidence for a role of TGF-β in tumor invasion and/or metastasis (1–6). Now, two papers in this issue of the *JCI* highlight this clinically significant action of TGF-β in tumorigenesis and provide very encouraging results regarding both the efficacy and the low toxicity of a soluble TGF-β receptor antagonist that effectively reduces tumor spread (7, 8).

Positive and negative effects of TGF-β signaling in cancer

TGF-β is a potent growth inhibitor of all epithelial and hematopoietic cells and can also induce apoptosis (1–3). For this reason, much emphasis has been placed on elucidating TGF-β signaling pathways, particularly those responsible for growth inhibition (summarized in Figure 1). After activation of the TGFβ type II/TGFβ type I (TβRII/TβRI) receptor complex, TGF-βs signal predominantly via the Smad pathway, although the activated receptor complex can also signal independently of Smads, via phosphatidylinositol 3-kinase (PI3K), protein phosphatase 2A/p70 S6 kinase (PP2A/p70S6K), and various mitogen-activated protein kinase (MAPK) pathways. There is also interplay between these pathways, such that activation of the Ras pathway or other non-Smad pathways can modulate signaling via Smads (1–6).

Homozygous mutations or deletions in the genes for Smad4, TβRII, or Smad2 are observed in some human tumors (1–3), suggesting a significant role for TGF-β signaling in tumor suppression. Nevertheless, only a minority of tumors show this type of genetic aberration, and the most commonly deleted such gene, *MADH4* (encoding Smad4), is not essential for all TGF-β activities (1–3). Some authors have suggested that the tumor-suppressing function of *MADH4* can be attributed to its antiangiogenic effect (not necessarily mediated by TGF-β), rather than to growth inhibition (9).

The tumor-suppressive effects of TGF-β have been clearly demonstrated in transgenic mouse models. Hemizygous or homozygous *Tgfb1*-null animals show an increased incidence of chemically or spontaneously induced tumors, respectively (1–3, 10, 11). Similarly, targeting a dominant negative TβRII to mammary or skin epithelia also enhances tumorigenicity, whereas TGF-β1–overexpressing mice have a decreased incidence of tumors (1–3). This tumor-suppressive function of TGF-β has raised concerns about the use of TGF-β antagonists to treat cancer, despite the increasingly strong evidence that TGF-β1 can promote tumor metastasis.

It is widely accepted that during multistage tumorigenesis, TGF-β growth-inhibitory and apoptotic effects are lost, frequently by subversion of the normal signaling pathway due to activation of other signaling molecules including PI3K and Ras (1–3). Meanwhile, other TGF-β responses prevail, unrelated to

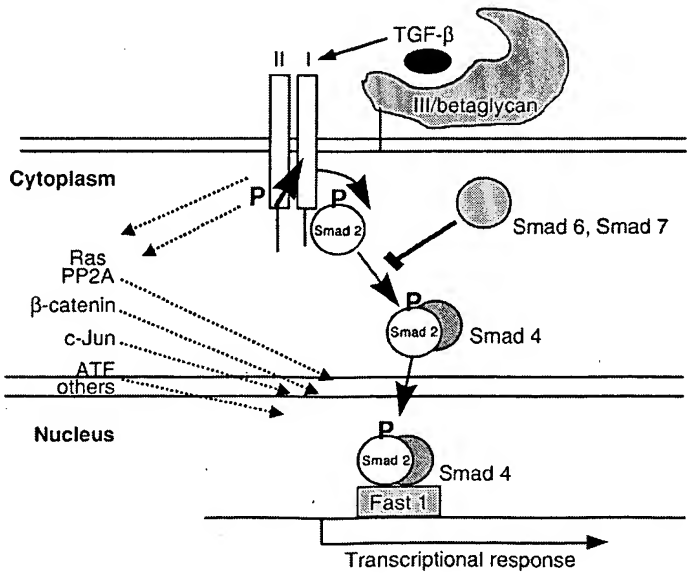

Figure 1
The TGF-β signaling pathway. TGF-βs bind and activate the TGF-β receptor complex, which transmits signal predominantly via activation and nuclear translocation of Smad proteins. However, several Smad-independent signaling pathways are also activated by this receptor complex, and the outcome of Smad signaling can be modified by interaction with other signaling pathways (1).

growth inhibition and favoring tumorigenesis (Figure 2; refs. 1–3). Moreover, as tumor cells progress, they secrete ever-increasing quantities of TGF-β1 (1–3, 6). TGF-β activity is increased partly by autostimulation of the *Tgfb1* gene, but also through transcriptional activation by Ras and other effectors, as well as by the action of proteases that activate the latent TGF-β in the ECM (1–3, 6).

In response to elevated TGF-β levels, the tumor cell becomes more migratory and invasive. Indeed, in cooperation with activated Ras, TGF-β1 can induce a complete epithelioid-to-fibroblastoid transition in both mammary and keratinocyte-derived tumors (1–3, 6), and it can drive metastasis of epithelioid tumors (6–8, 12). TGF-β can also stimulate tumor angiogenesis, alter the stromal environment, and cause local and systemic immunosuppression, all of which contribute to tumor progression and metastasis (1–3).

As discussed in the two articles in this issue of the *JCI* (7, 8), the concept of using soluble protein antagonists that bind and inactivate extracellular TGF-β was first tested over a decade ago using decorin, a natural inhibitor of TGF-β, in a therapeutic model for fibrosis (8). More recently, the chimeric Fc:TβRII protein used in the current studies has proved attractive because of its high affinity for TGF-β, its ready purification by protein A affinity chromatography, and its effectiveness in a number of models of fibrosis.

Early attempts to demonstrate the efficacy of this approach involved stably transfected glioma (13), thymoma (14), pancreatic (15), or metastatic breast tumor cell lines (16) carrying cDNAs for soluble forms of decorin (13), TβRII (14, 15), or TβRIII (16). Each demonstrated tumor suppression after subsequent injection of the modified tumor cell line into mice. In the first two cases (13, 14), this was attributed to re-acquisition of tumor-specific cellular immunity, whereas the effects on the pancreas and breast cancer lines included suppression of invasion (15), angiogenesis (15), and lung metastasis (16).

Efficacy and toxicity

The articles in this issue of the *JCI* (7, 8) have pushed the story two steps further, firstly by applying soluble Fc:TβRII as an injectable drug to prove efficacy in suppression of breast tumor metastasis in vivo (7), and secondly by screening for any adverse effects on the mice after lifetime exposure to high-level circulating Fc:TβRII (8). Muraoka et al. (7), using the MMTV-PyV mT transgenic model of mammary tumorigenesis, show that twice-weekly intraperitoneal injection of Fc:TβRII reduces lung metastasis tenfold. Fc:TβRII treatment also inhibits metastasis of two metastatic mammary cell lines. In all three cases, Fc:TβRII has no effect on proliferative rate of the primary tumor cells. Yang et al. (8) take a different approach, focusing on possible adverse effects in transgenic mice that stably express soluble Fc:TβRII. Circulating Fc:TβRII, which is found at about 1 mg/ml in the blood, not only reduces metastasis formation of melanoma cells injected into the tail vein of the mice but also reduces metastasis to the lung from endogenous mammary tumors that arise when the mice are crossed onto the MMTV-Neu transgenic model of mammary carcinogenesis. Both groups find that Fc:TβRII leads to no changes in tumor latency, yield, or size.

Taken together, the two papers (7, 8) show that soluble Fc:TβRII is efficacious in reducing tumor metastasis, whether delivered genetically from within the neoplastic cell or administered as an injectable circulating drug. Both groups also addressed the mechanisms of action of Fc:TβRII in attenuating metastatic spread. In the MMTV-PyV mT model, Muraoka et al. (7) specifically exclude an effect on TGF-β–induced angiogenesis. In their model, Fc:TβRII appears to decrease tumor cell intravasation and/or decrease survival of tumor cells in the circulation, since the number of circulating tumor cells is lower in the Fc:TβRII-treated mice than in controls (7). In support of this mechanism, Smad2 activation has recently been shown to drive tumor cell extravasation in a skin tumor model (6). Consistent with an effect on tumor intravasation, the Fc:TβRII-treated mammary tumor cells are altered toward a more differentiated, less motile/migratory phenotype than is seen in untreated tumor cells. Production of active matrix metalloproteinase 2 (MMP2) and MMP9, proteases thought to be important in tumor invasion, migration, and intravasation, is diminished and apoptosis is elevated in response to Fc:TβRII (7).

In the injectable melanoma model, Yang et al. (8) argue, the effect of Fc:TβRII on metastasis is likely indirect, possibly including decreased angiogenesis and/or elevated immunosuppression. Although metastasis is diminished in the Fc:TβRII transgenic mice following tail vein injections of melanoma cells, the initial appearance of micro-metastases

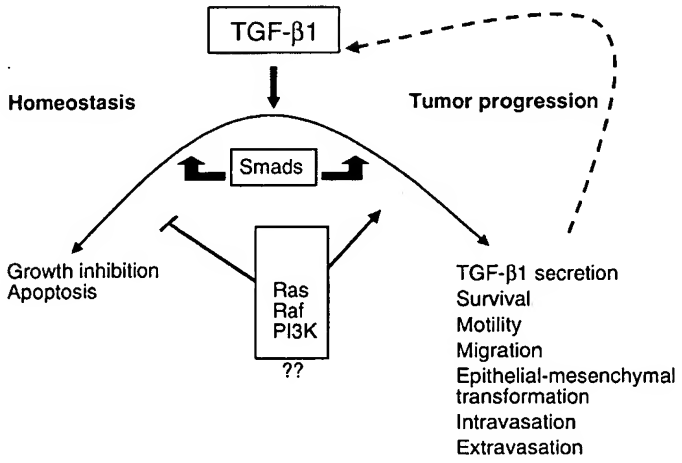

Figure 2
The balance between the autocrine homeostatic and tumor-progressing activities of TGF-β is perturbed by activation of oncogenic signaling pathways. As tumor progression proceeds, the homeostatic branch of TGF-β action becomes increasingly compromised, and tumors secrete more TGF-β1, thus exacerbating tumor progression.

is no different from that seen in wild-type mice. Since TGF-β has multiple actions that can drive tumor metastasis, the exact mechanisms involved are probably context-specific, depending on the tumor type, genetic constitution, and the exact stage of carcinogenesis of the tumor. However, the exciting take-home message is that soluble TGF-β antagonists can significantly decrease metastasis in models of breast cancer and melanoma, as previously suggested for thymoma (13), glioma (14), and pancreatic (15) and mammary carcinoma (16).

The complete absence of TGF-β1 in mice leads to death resulting from uncontrolled systemic inflammation, and even a T cell–specific deficit in TGF-β causes lethal immune system defects (discussed in ref. 8). Therefore, the apparent absence of side effects, even after lifetime exposure to approximately 1 mg/ml circulating Fc:TβRII (8), is particularly encouraging. No immune phenotype was seen in the study of Yang et al. (8), apart from a minimal increase in memory T cells, and a nonsignificant increase in lymphocytic infiltration into organs of aged mice. The authors employed several techniques to demonstrate that Fc:TβRII at this level does not completely block all TGF-β1 bioactivity in vivo (8). Indeed, although circulating TGF-β levels are reduced in the Fc:TβRII transgenics to probably ≤10% of the wild-type level, these animals still thrive in the laboratory. It would be interesting to examine how the mice fare when challenged with other environmental hazards such as foreign antigens and pathogens.

Also heartening is the finding by both groups that soluble Fc:TβRII had no tumor-promoting action in vivo. Conversely, mice in which TGF-β activity is diminished globally, as a result of hemizygosity for *Tgfb1*, are tumor-prone (10, 11), as are animals in which this factor is ablated in a tissue-specific manner using dominant negative (DN) TβRII. The basis of this discrepancy is uncertain, but it may be that Fc:TβRII preferentially targets circulating TGF-β1 because it is too bulky to gain access to the more functionally important TGF-β tightly bound to the cell surface or ECM. In addition, different thresholds of TGF-β activity required for the growth-suppressing and the metastasis-promoting effects of TGF-β could help account for the tumor incidence seen in DN-TβRII transgenic strains. Growth inhibition, for example, is more sensitive than other TGF-β responses to decreases in the level of TβRII (3, 17). Moreover, recent studies in a skin carcinogenesis model do indeed show that a high threshold of Smad2 activation must be surpassed in order to drive metastatic spread (6).

Nevertheless, since *Tgfb1*$^{+/-}$ animals have an increased incidence of chemically induced tumorigenesis (10), one might expect a similar phenotype in Fc:TβRII mice (8), but this is not the case. The explanation probably lies in the different models used. The tumors and cell lines studied by Muraoka et al. (7) have already lost growth sensitivity to TGF-β, as assessed by BrdU incorporation, so tumor-suppressive effects of TGF-β would not be expected. The TGF-β growth sensitivity of MMTV-Neu tumor cells has not been studied, but transfection of normal differentiated thyroid cells with ErbB2 (Neu) attenuates the growth-inhibitory response to TGF-β, suggesting that Neu does indeed attenuate growth sensitivity to TGF-β (18). In this context, chemical carcinogenesis studies on Fc:TβRII transgenic mice are warranted to uncover any tumor-promoting effects of Fc:TβRII, especially in view of the fact that a soluble TβRII transfected into a hepatoma cell line has been shown to promote tumor development (19).

Despite these reservations, Fc:TRII clearly is highly efficacious in reducing metastasis and is of exceptionally low toxicity in mice. Indeed, many drugs for treatment of both malignant and nonmalignant conditions, such as cyclosporin, have tumor-promoting activity (12), and most cancer drugs show general cytotoxicity levels orders of magnitude higher than does this soluble TβRII receptor.

Future developments in anti-TGF-β drug design

Pharmaceutical companies have avoided TGF-β agonists or antagonists, partly because of fear of nonspecificity and consequent side effects. The articles in this issue of the *JCI* (7, 8) might cause them to reconsider this decision. TGF-β antagonists such as Fc:TβRII could prove as useful clinically as Herceptin (20), an anti-Neu antibody used for the treatment of Neu-positive breast tumors. They would also be expected to have a wider range of applications, since metastasis of many tumor types may be inhibited by their use.

Small-molecule inhibitors of TGF-β action could also be of value and should offer better drug specificity than the fusion protein described here. Their design will depend on a greater understanding of the cross-talk between the intracellular signaling pathways that propagate TGF-β metastatic versus homeostatic signals in different cell and tumor types (Figure 2). However, it should be possible to design and select small-molecule drugs that specifically inhibit the invasion/metastasis branch of TGF-β action, while leaving growth-inhibitory and apoptotic pathways intact. Inhibition of the Ras/Raf and/or PI3K pathways, in addition to blocking the cell survival and mitogenic effects of these pathways, might also attenuate the adverse effects of TGF-β (1). Hence, combination therapies using metastasis inhibitors that target TGF-β, as well as specific Ras/Raf and/or PI3K inhibitors, might be particularly efficacious and safe.

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Reversal of tumor-induced immunosuppression by TGF-β inhibitors

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Key words: TGF-β, TGF-β inhibitors, malignancy, cancer, immunosuppression

Summary

The immune system is responsible for the early detection and destruction of newly transformed malignant cells. Some transformed cells become immunologically invisible by passive avoidance of immune surveillance (i.e., when tumor cells are immunologically indistinguishable from normal cells). Other transformed cells actively secrete cytokines that effectively blind the immune system to the presence of abnormal antigens on the tumor cell surface. Transforming growth factor-β ("TGF-β"), which is expressed by a majority of malignant tumors, is the most potent immunosuppressor and therefore, the most likely cytokine to be responsible for the latter phenomenon. In addition to playing a key role in tumor-induced immunosuppression, TGF-β stimulates angiogenesis. Interestingly, tumor cells eventually become refractory to TGF-β-mediated growth arrest, either due to loss of TGF-β receptors or due to dysregulation in TGF-β signaling pathways. Neutralization of TGF-β or inhibition of its production is an effective method of cancer treatment in variety of animal models. Several agents targeting TGF-β are in the early stages of development and include anti-TGF-β antibodies, small molecule inhibitors of TGF-β, Smad inhibitors and antisense gene therapy. Since tumors may express more than one isoform of TGF-β, these new drugs should target all three TGF-β isoforms produced by human tumors. The effects of therapies targeting TGF-β are likely to be synergistic with cytotoxic chemotherapy and immunotherapy. Reversal of TGF-β-induced immunosuppression is a new and promising approach to cancer therapy, with potential applications in other diseases such as AIDS.

Transforming growth factor-β pathway

Transforming growth factor-β ("TGF-β") belongs to a superfamily of structurally related regulatory proteins, which include activin/inhibins, bone morphogenic proteins ("BMPs") and Müllerian inhibiting substance [1–4]. Three isoforms of TGF-β: TGF-β1, TGF-β2 and TGF-β3 are produced by mammals. These molecules are closely related and have a high degree of homology [2].

The majority of human tissues can express TGF-β. Platelets are the richest source of this cytokine [5]. TGF-β is usually secreted as an inactive, latent complex ("LTGF-β") containing two propeptide residues which are cleaved off during the activation process: latency associated protein ("LAP") and latent TGF-β binding protein ("LTBP") [6]. The latter, together with hyaluronic acid which protects LTGF-β from tryptic degradation, may play a significant role in matrix storage of latent TGF-β [7,8]. Factors which control the transformation of the latent to the biologically active molecule are the primary regulators of TGF-β activity. Active TGF-β is cleaved from the latent complex by plasmin-dependent and plasmin-independent pathways [9]. Thrombospondin, present in platelets and in extracellular matrix protein, is a TGF-β activator [10]. Tumor cells produce proteases (i.e., serine protease), which activate LTGF-β [11,12]. Metalloproteinases MMP2 and MMP9, which are frequently expressed by malignant cells, especially at sites of tumor cell invasion, can also activate LTGF-β [13,14].

TGF-β signaling involves the interactions of at least three different receptors. Types I (RI) and II (RII) receptors are transmembrane serine/threonine kinase receptors, which form ligand-mediated heteromeric complexes. Type III receptor (RIII) modulates ligand binding to the RI and RII signaling complex

[15,16]. TGF-β binds first to RII, which recruits RI and leads to its phosphorylation [17,18]. Activation of these receptors mediates the cellular effects of TGF-β.

Several molecules participate in the intracellular TGF-β signaling cascade. These include protein kinase C, phospholipase C, protein phosphatase 1, Ras, mitogen-activated protein kinases, stress-activated protein kinases and the Smads (Sma and Mad homologues) [19–22].

Smads are probably the most important signaling components for several members of the TGF-β superfamily [23–25]. Smad1 and Smad5 are relatively specific for BMP signaling whereas Smad2 and Smad3 mediate TGF-β and activin signaling [21,22]. The working model for Smad regulation proposes a sequence of events that occur after receptor activation. These include: phosphorylation of pathway-restricted Smads; heteromeric complex formation of these Smads with Smad4; and translocation to the nucleus resulting in activation of gene transcription [26,27]. TGF-β also regulates expression of inhibitory Smads (Smad6 and Smad7) [28,29].

While Smad-dependent pathway is very important for TGF-β signaling, it is difficult to identify a common target for all pathways downstream of TGF-β receptors and indeed different cellular functions induced by TGF-β, i.e., growth arrest, apoptosis and epithelial to mezenchymal transition may be mediated through distinct effector functions, requiring either Smad-dependent or Smad-independent pathway, or both [30].

Active immunotherapy is one of the most promising approaches to cancer treatment. However, stimulation of the immune effector cells and/or improved tumor antigen presentation do not necessarily ensure a meaningful therapeutic outcome. Tumor-induced immunosuppression is the most likely reason for the disappointing results of studies exploring this therapeutic approach [31]. Passive avoidance of immune surveillance may play a role in preventing recognition and destruction of some tumor cells, but tumors also protect themselves actively from the immune system. TGF-β can provide such protection for established tumors.

Role of TGF-β in tumor progression and metastasis

Common tumors overexpressing TGF-β include breast cancer [32,33], prostate cancer [34,35], small and non-small cell lung cancer [36–38], colorectal cancers [39], pancreatic cancer [40], ovarian cancer [41], bladder cancer [42], Kaposi sarcoma [43], malignant melanoma [44] and malignant gliomas [13] (Table 1).

Animal studies suggest that promotion of invasion and metastasis is the principal *in vivo* activity of TGF-β [45–48]. In unpublished experiments by Michael O'Reilly in Judah Folkman's laboratory, systemic administration of TGF-β stimulated the growth of microscopic dormant tumor metastases of murine melanoma B16F10. Systemic administration of bFGF potentiated the growth stimulatory effect of TGF-β. However, bFGF administered alone was only weakly effective compared to TGF-β alone (Folkman J: personal communication, 2001).

In addition to producing TGF-β malignant tumors may activate LTGF-β derived from surrounding tissues or platelets [10]. Many common tumors can induce intravascular clotting, particularly within tumor vasculature. Platelets constitute one of the largest storage pools of TGF-β, storing it in very high concentrations and releasing it during degranulation [49]. An inverse relationship between plasma TGF-β levels and platelet counts has been observed in some patients with malignant tumors; TGF-β levels increase as platelet counts drop [50]. This observation suggests that increased plasma TGF-β levels may

Table 1. Common tumors overexpressing TGF-β

Tumor type	References
Breast cancer	[32,33]
Prostate cancer	[34,35]
NSCLC	[38]
SCLC	[36,37]
Colorectal cancer	[39]
Pancreatic cancer	[40]
Ovarian cancer	[41]
Cervical cancer	[121]
Bladder cancer	[42]
Kaposi sarcoma	[43]
Malignant melanoma	[44]
Malignant gliomas	[12]
Renal cell carcinoma	[122]
Multiple myeloma	[123,124]
Head and neck cancer	[125]
Papillary thyroid carcinoma	[126]
Esophageal cancer	[127]
Gastric cancer	[128]
Hepatocellular carcinoma	[129]

reflect intravascular clotting rather than increased tumor secretion of TGF-β in some tumor-bearing hosts.

Many cellular effects of TGF-β facilitate tumor growth and metastasis [51–53]. Both lymphocytes and tumor cells express TGF-β receptors. In contrast with immune cells, tumor cells eventually cease to express TGF-β receptors, become insensitive to inhibitory effects of TGF-β and therefore acquire invasive and/or metastatic phenotype [54,55]. Consequently, TGF-β does not inhibit tumor growth, but produces immunosuppression in patients with advanced or metastatic tumors.

In addition to dampening the immune response, TGF-β promotes angiogenesis both directly and indirectly [56], regulates the expression and secretion of various types of collagen (affecting cell adhesion and migration) [57] and modulates the response to epidermal growth factor [58].

Animal experiments suggest that TGF-β-mediated immunosuppression is the most important of these effects and that the presence of activated TGF-β in the tumor microenvironment protects tumor cells from recognition by the immune system [59]. While the exact mechanism of this phenomenon is not clear, in an *in vivo* murine colon carcinoma model, animals bearing a tumor for periods longer than 26 days developed CD8+ T-cells with impaired cytotoxic function and decreased ability to mediate an antitumor response *in vivo* [60]. T lymphocytes from these mice expressed T cell antigen receptors that contained only low amounts of CD3 gamma and in which the CD3 zeta chain was replaced by the Fc epsilon gamma chain. These changes could explain TGF-β-induced immune defects in tumor-bearing hosts.

Interestingly, *in vivo* injection of genetically modified tumor cells which were unable to secrete TGF-β, resulted in the destruction of TGF-β-producing tumor deposits at distant locations [31]. This study suggested that immune recognition cells are much more sensitive to the inhibitory effects of TGF-β than are immune effector cells. However in other studies, TGF-β inhibited *in vitro* activation and subset expansion of activated effector cells [61]. It also impaired the function and expression of high-affinity IL-2 receptors and altered antigen-specific T-cell responses [62,63]. Further studies demonstrated the ability of TGF-βs to inactivate natural killer ("NK") and lymphokine activated killer ("LAK") cells, probably by inhibition of TNF-α and -β secretion [64,65]. Additionally, TGF-β is involved in the generation of CD8+ T-suppressor cells and it completely abrogates synthesis of immunoglobulin G *in vitro* [66,67]. Consequently, this cytokine can interfere with both the recognition and destruction of tumor cells by the immune system.

Treatment with anti-TGF-β antibodies suppressed the development of primary tumors and the formation of distant metastases in nude mice bearing one of two types of human colon adenocarcinomas SLU-1 or SLU-M1. It is of interest that anti-TGF-β antibodies suppressed SLU-M1 proliferation *in vitro*, whereas SLU-1 proliferation was not affected by either anti-TGF-β antibodies or exogenous TGF-β1. Both of these cell lines had a very similar metastatic potential *in vivo* in untreated animals [68]. These findings suggest that tumor growth and metastasis are not significantly affected by interactions between TGF-β and receptors on the tumor cell surface, and that the primary target of TGF-β *in vivo* is the immune system and not the tumor itself.

Neutralization of TGF-β as a therapeutic strategy

The preclinical data and the ability of a majority of human malignant tumors to secrete TGF-β suggest that inhibitors of TGF-β may be useful in treating a wide variety of human malignancies. Figure 1 depicts potential targets for therapy aiming to disrupt TGF-β-mediated immunosuppression. A number of new agents targeting TGF-β, its receptors or its intracellular pathways are currently being investigated (Table 2).

TGF-β-binding proteins

A variety of naturally occurring proteins effectively inhibit TGF-β. Fetuins are globular glycoproteins expressed in multiple tissues during mammalian embryogenesis [69]. A human homologue of fetuin, α2-HS glycoprotein, is secreted by the liver and accumulates in the bones [70]. Fetuin binds to TGF-β1 and TGF-β2, but it has much higher affinity for bone morphogenic proteins (BMP-2, BMP-4 and BMP-6) [71]. While the binding of fetuin to TGF-β results in inactivation of the latter molecule [72], its lack of specificity and relatively large molecular size preclude its use in the clinical setting.

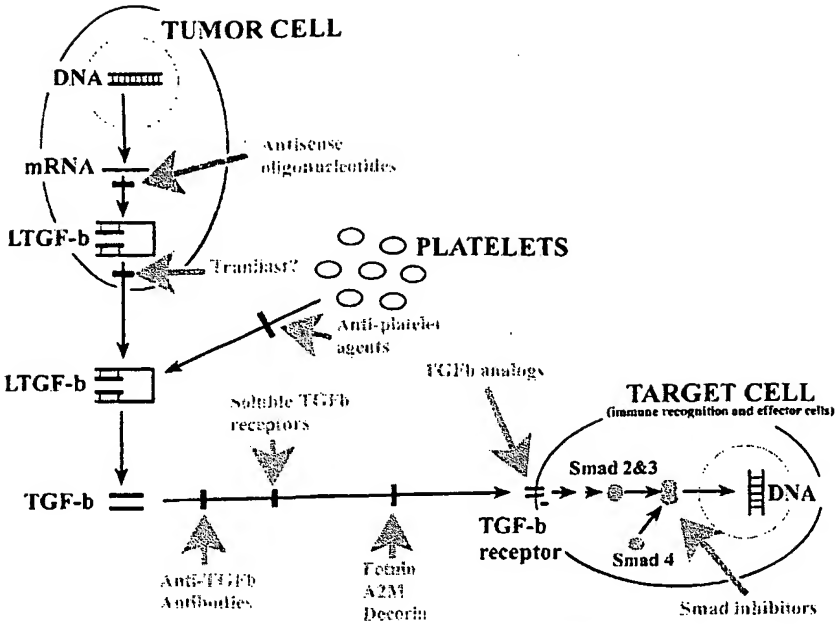

Figure 1. Potential targets for therapy aiming to disrupt TGF-β-mediated immunosuppression.

Table 2. TGF-β inhibitors in development

Agent	Category	TGF-β isoforms targeted	Company	Stage of development	Indication	References
CAT-192	Humanized monoclonal antibody	TGF-β1	Genzyme/CAT	Phase II	Diffuse systemic sclerosis	[130]
CAT-152 (lerdelimumab)	Humanized monoclonal antibody	TGF-β2	Genzyme/CAT	Phase II	Prevention of scarring following surgery for glaucoma	[131]
1D11	Murine monoclonal antibody	TGF-β1 TGF-β2 TGF-β3	Genzyme/CAT	Preclinical	Diffuse scleroderma, radiation-induced fibrosis	[132]
2G7	Murine monoclonal antibody	TGF-β1 TGF-β2 TGF-β3	Genentech	Preclinical	Malignancy	[45]
Tranilast (Rizaben)	Small molecule	TGF-β1 TGF-β2	Kissei Pharma	Phase III	Allergic rhinitis, Prevention of restenosis following PTCA	[88]
AP-12009	Antisense oligonucleotide	TGF-β2	Antisense Pharma	Phase I/II	Glioblastoma Pancreas	[98]

A shorter sequence (18–19 amino acids) with homology to the TGF-β receptor type II (TβRII) was identified within the fetuin molecule and was designated as TGF-β receptor type II homology 1 domain (TRH1). However, cyclized TRH1 peptides from fetuin bind preferentially to BMPs, suggesting their limited utility as inhibitors of TGF-β. Interestingly, cyclized TRH1 peptides originating from TβRII bind with higher affinity to TGF-β1 than to BMP-2 and therefore merit further studies [63].

Human alpha 2-macroglobulin ("α_2M") is a plasma protein and cytokine carrier that binds isoforms in the TGF-β family. Activated α_2M selectively neutralizes the immunosuppressive effects of TGF-β and promotes the activation of NK, LAK and tumor-specific CTL responses [73]. However, this molecule is too large to be useful for clinical applications. It would be reasonable to identify a more specific TGF-β-binding sequence within the structure of α_2M that can be isolated for further development.

Decorin is a small chondroitin-dermatan sulfate proteoglycan that binds selectively to TGF-β and appears to inhibit its synthesis [74]. Two other proteoglycans of the decorin family, biglycan and fibromodulin, also selectively bind to TGF-β [75]. Decorin appeared to increase the binding of TGF-β to its receptors in one study and therefore authors postulated that it enhanced the cellular effects of TGF-β [76]. However, decorin inhibited the growth of malignant glioma in an animal model, suggesting that its predominant effect *in vivo* is inhibition of TGF-β [77].

TGF-β receptor-related inhibitors and Smad inhibitors

The TGF-β type III receptor, also known as betaglycan, is a membrane-anchored-proteoglycan that presents TGF-β to the type II signaling receptor. The extracellular region of this receptor may be shed by cells into the medium. Soluble betaglycan binds TGF-β, but does not enhance binding to membrane receptors. In effect, recombinant soluble betaglycan acts as a potent inhibitor of TGF-β binding to membrane receptors and blocks TGF-β action. This effect is particularly pronounced with the TGF-β2 isoform [78]. Treatment with recombinant TGF-β type III receptor (soluble RIII) inhibited angiogenesis and tumor growth in human breast cancer xenografts and significantly reduced number of metastases in the lung and axillary lymph nodes in this model [79]. Soluble TGF-β receptor II appears to have similar properties and was shown to suppress tumorigenicity in a murine tumor model [80]. Constitutional expression of a soluble TGF-β antagonist, which incorporates in its structure extracellular domain of type II receptor, protects against metastasis in a murine model [81]. Inhibitors targeting TGF-β type I receptor serine–threonine kinase appear to have similar effects [82].

Smad proteins have recently been identified as important mediators of the responses to TGF-β and related factors. Smad4 (the product of the tumor suppressor gene DPC 4) cooperates with Smad1, Smad2 and Smad3 and acts as a common mediator of signaling by the TGF-β family of molecules [83]. Smad inhibitors block intracellular TGF-β signaling pathways and therefore may prevent TGF-β-induced inactivation of immune cells. Several studies demonstrate the feasibility of targeting intracellular TGF-β pathways [84]. There are no published studies with small molecule Smad inhibitors. However, activation of an isopropyl-1-thio-beta-d-galactopyranoside-inducible system to express Ha-Ras(Val-12) in intestinal epithelial cells caused a decrease in the level of Smad4 expression, inhibited TGF-β-induced complex formation between Smad2/Smad3 and Smad4, blocked Smad4 nuclear translocation, inhibited the TGF-β-mediated decrease in [(3)H]thymidine incorporation, and repressed TGF-β-activated transcriptional responses [85].

Smad6 or Smad7-derived molecules could be used to target this pathway. Phosphorylation of Smad2/Smad3 by activated RI is inhibited by Smad6 and Smad7. Stable transfection of COLO-357 human pancreatic cancer cells with a full-length Smad7 construct led to a complete loss of the growth inhibitory response to TGF-β1 [86].

TGF-β-binding peptides

Latency-associated peptide inhibits all three isoforms of TGF-β *in vitro* as well as after intraperitoneal administration in a murine model [87]. LAP is readily absorbed from the peritoneal cavity and reaches sufficient concentrations in tissues to inhibit TGF-β. This compound enhanced antigen-specific T-cell proliferation and gamma interferon mRNA expression in another *in vivo* model [88] however.

Monoclonal anti-TGF-β antibodies

A limited number of animal studies with antibodies targeting TGF-β have been conducted to date. In one experiment, intraperitoneal administration of monoclonal anti-TGF-β antibodies effectively abrogated tumor formation in mice inoculated with MCF-7 breast cancers [45]. The response to treatment with anti-TGF-β antibody was dose dependent in this model. However, the effectiveness of this antibody was dramatically decreased when treatment was initiated later than 24 h after tumor inoculation. This observation suggests that targeting TGF-β alone may not be sufficient to achieve meaningful therapeutic

effects in patients who present with advanced or metastatic disease.

Indeed, another study confirmed that monotherapy targeting TGF-β is not necessarily the optimal treatment for more advanced, aggressive tumors [46]. Mice injected with a highly metastatic strain of B16 melanoma were treated with either IL-2 or monoclonal anti-TGF-β antibody alone. While treated mice had fewer metastatic lesions compared with control animals, the difference was not statistically significant. However, the number of lung metastases was decreased threefold (a statistically significant difference) when mice were treated with a combination of the anti-TGF-β antibody and IL-2. These results suggest that the combination of IL-2 and anti-TGF-β antibody has synergistic antitumor effects and that therapies targeting TGF-β should be investigated in combination with other, established antitumor agents. Two other studies using IL-2 in combination with anti-TGF-β antibody did not demonstrate significant antitumor effects due to the inadequate dose of anti-TGF-β antibody used [89,90].

Cyclosporin induced invasive behavior in adenocarcinoma cells *in vitro* and enhanced tumor growth in immunodeficient SCID-beige mice [91]. Anti-TGF-β antibodies prevented the cyclosporin-induced increase in metastases. This study, as well as the one cited above, suggest a role for TGF-β inhibitors in the adjuvant setting.

Regulation of TGF-β expression

Kissei Pharma has developed and launched tranilast (*N*-[3,4-dimethoxycinnamoyl]-anthranilic acid) in Japan and South Korea for the treatment of allergic rhinitis, asthma and atopic dermatitis. Kissei, in collaboration with GlaxoSmithKline, has been developing tranilast (as Rizaben) for the prevention of restenosis following percutaneous transluminal coronary angioplasty (PTCA). Tranilast has an acceptable safety profile when administered PO at doses up to 600 mg/day for up to 3 months and it has been studied in over 11,500 patients worldwide for a variety of indications [92].

Tranilast inhibits transcriptional mechanisms associated with the upregulation of TGF-β and its receptors [93]. In cell cultures it inhibited TGF-β1 and TGF-β2 secretion and it antagonized the effects of TGF-β on cell migration and proliferation by blocking chemotactic responses and tumor cell invasiveness [94]. These effects were observed at tranilast concentrations that did not produce direct cytotoxicity. Interestingly, tranilast did not affect $\alpha_v\beta_3$ integrin expression at the cell surface but inhibited matrix metalloproteinase-2 expression and activity. Tranilast inhibited antibody-mediated hypersensitivity reactions as well as IFN-γ and IL-2 production [95]. This compound also inhibited VEGF-induced angiogenesis and vascular permeability in a dose-dependent manner [96,97].

Tranilast demonstrated antitumor activity in a number of *in vitro* and *in vivo* models. Oral administration of tranilast inhibited the growth of experimental 9L rat gliomas and reduced the expression of TGF-β2 *in vivo* [98]. The proliferation of the human scirrhous gastric cancer cell line, OCUM-2M, was inhibited by tranilast in an *in vitro* assay (in a coculture with fibrocytes) as well as in an *in vivo* gastric carcinoma model, when tranilast was administered alone [99] or in combination with cisplatin [100]. Tranilast also inhibited growth of breast cancer cell line MCF-7 *in vitro* [101] and had antitumor and antiangiogenic effects in an murine Lewis lung cancer model [102]. In the latter study, tranilast potentiated the inhibition of the tumor growth induced by cyclophosphamide, *cis*-diamminedichloroplatinum(II), adriamycin and vindesine.

Antisense oligonucleotides targeting TGF-β2 DNA or mRNA inhibited malignant mesothelioma growth *in vitro* and *in vivo* [103]. TGF-β antisense gene therapy administered to rats inoculated intracerebrally with gliosarcoma significantly prolonged survival compared to IL-2 gene therapy or controls [31]. All of the rats vaccinated with TGF-β antisense modified tumor cells survived for a period of 12 weeks after tumor implantation, while survival in control animals ranged from 0% to 30% in the same observation period. Even more interesting was an apparent complete eradication of CNS tumors (confirmed by pathologic examination) in animals treated with antisense TGF-β vaccine [31]. This approach is currently being tested in Phase I studies with encouraging early results [104].

Another study investigated a TGF-β antisense plasmid vector (pCEP4/TGF-β antisense) alone and in combination with IL-2 gene therapy in an intraperitoneal model of murine ovarian teratoma (MOT) [105]. MOT cells, like many human ovarian carcinomas, produce TGF-β. Production of TGF-β by MOT cells was suppressed in this study which utilized the above-mentioned vector. Subsequent subcutaneous immunization of C3H mice with a mixture of IL-2

gene-transduced fibroblasts and TGF-β antisense-modified MOT cells induced significantly better protection against a subsequent intraperitoneal tumor challenge compared with immunization with unmodified MOT cells alone (11/16 (69%) vs. 4/21 (19%) tumor-free animals, $P < 0.01$). Immunization with either a mixture of IL-2 gene modified fibroblasts and unmodified MOT cells (2/12 (17%) tumor-free animals) or TGF-β antisense-modified MOT cells alone (0/13 tumor free animals) failed to induce significant protection compared with immunization with unmodified MOT cells. These data show that combined TGF-β antisense and IL-2 gene therapy is required to generate effective antitumor responses in the MOT model.

Some antisense compounds are already being evaluated in early clinical studies. Antisense Pharma initiated Phase I/II trials of intratumoral administration of AP-12009, a phosphorothioate TGF-β2-specific antisense oligonucleotide for the treatment of brain tumors (glioblastoma) [106,107]. Preclinical studies demonstrated that AP-12009 prevented proliferation of glioma cells *in vitro* and reversed T-cell immunosuppression caused by TGF-β in MLTC systems [98].

Discussion

Reversal of TGF-β-induced immunosuppression is a new and promising approach to cancer therapy, with potential applications in a variety of other diseases, including AIDS [108]. Since most, if not all, malignant tumors produce immunosuppressive factors, neutralization of these molecules should become one of the key targets of anticancer therapy.

Animal studies identified TGF-β as the cytokine most likely to be responsible for tumor-related immunosuppression and, in effect, for maintenance of the malignant state, tumor growth and metastasis. These studies confirmed the hypothesis that reversal of tumor-induced immunosuppression affects tumor growth and metastasis and that targeting TGF-β may lead to complete tumor eradication. Since tumors may utilize TGF-β from other sources such as platelets or interstitial cells, antiplatelet agents and/or anticoagulants may play a contributory role in the treatment of TGF-β-dependent cancers.

Since TGF-β appears to block tumor-specific immunity, approaches that disrupt TGF-β signaling in T-cells and other immune cells may offer yet another approach to cancer immunotherapy [109,110]. T-cell -specific blockade of TGF-β signaling resulted in the enhancement of antitumor immunity and induced an immune response capable of eradicating tumors in mice challenged with live tumor cells [111]. Another study demonstrated that activation of T-cells by IL-15 renders these cells resistant to suppression by TGF-β. Moreover, IL-15 treatment restored proliferative ability of T-cells that were already exposed to TGF-β [112].

Findings in some studies suggest that targeting TGF-β alone may not be sufficient to eradicate established tumors. Administration of anti-TGF-β antibodies to tumor bearing animals increased drug sensitivity to some of the widely used chemotherapeutic agents, such as cyclophosphamide and cisplatin [113]. This study suggests that the sequence of treatments may be important when TGF-β inhibitors are studied in combination with other tumor-targeting agents. However, additional studies evaluating the effects of treatment sequence are needed to determine whether administration of TGF-β inhibitors should precede or follow other therapies. *In vitro* studies demonstrated that decorin effectively inhibited the growth of two ovarian cancer lines, SKOV3 and 2774. Decorin has synergistic effects against ovarian tumor cells when administered with carboplatin [114]. For these reasons, TGF-β-targeting agents should be studied in combinations with chemotherapeutic agents, immunostimulating agents such as IL-2 or IL-12 and perhaps antiplatelet agents (to prevent platelet degranulation, decreasing levels of circulating TGF-β).

This new therapeutic approach may also have applications in the bone marrow transplant setting, where it could potentially achieve two therapeutic goals: eradication of residual tumor and shortening of the time necessary for bone marrow engraftment.

In patients with malignant tumors that can be treated with radiation therapy, i.e., non-small-cell lung cancer ("NSCLC") or head and neck cancer, increasing the dose of radiation may improve local control and perhaps even survival. However, the maximal dose of radiation is limited due to radiation-induced side effects such as pneumonitis and pulmonary fibrosis. TGF-β is one of the most fibrogenic cytokines and it plays a key role in radiation therapy-induced pneumonitis and fibrosis [115–117]. TGF-β inhibitors should be a very valuable addition to the treatment of these patients by achieving two therapeutic goals: targeting the tumor and preventing pneumonitis and fibrosis. Indeed, the small-molecule TGF-β inhibitor, tranilast, was

shown recently to prevent radiation-induced pneumonitis and fibrosis in an animal model [118]. One study suggests that until TGF-β inhibitors become widely available, measurements of plasma TGF-β levels may allow escalation of radiation therapy doses in patients with normal TGF-β levels, without increasing radiation-induced toxicities [119].

Some of the studies reviewed above suggest that the TGF-β plasma level could be used as "an universal marker" of tumor progression. Unfortunately, plasma concentrations of this cytokine can be confounded by the abundance of platelet-derived TGF-β and are not a direct measure of tumor cell TGF-β activity. Since plasma levels of TGF-β are significantly affected by the degree of platelet degranulation, intravascular clotting may affect the predictive value of TGF-β as a marker of tumor progression [120]. Artifactual contamination of plasma samples due to platelet degranulation can also occur easily during collection or processing of plasma samples. It would be useful to measure levels of markers of platelet degranulation in conjunction with TGF-β measurements to identify the sources of circulating TGF-β. Due to these problems, additional studies are needed to establish utility of this cytokine as a tumor marker.

No studies to date have investigated potential mechanisms of resistance to this novel tumor-targeting strategy. It is likely that the optimal spectrum of activity for drugs targeting TGF-β may have to encompass inhibition of all three isoforms of this cytokine, since tumor cells may increase production of the other two isoforms if only one is targeted. The success of novel targeted therapies is dependent, in part, on the degree of target inhibition that can be achieved at the tumor site. It is not clear whether some of the approaches to TGF-β neutralization (i.e., antibodies) can deliver a sufficient concentration of the studied compound within tumor tissue to achieve a therapeutic effect.

In conclusion, TGF-β is an important cytokine facilitating tumor growth and metastasis in malignant tumors. Preclinical studies demonstrated significant antitumor activity of agents targeting this molecule as well as synergy with immunostimulating agents and traditional chemotherapeutics. It is difficult to assess objectively which of the multiple effects of TGF-β contributes the most to tumor growth and metastasis. Clearly, agents that block TGF-β production, secretion, activation and metabolism should be extensively investigated as a new therapeutic modality for all types of solid tumors.

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